HEPATIC INSULIN RECEPTOR TYROSINE KINASE ACTIVITY IN DIABETES: MODULATION BY ASSORTED ADENOSINE TRIPHOSPHATASES/PHOSPHATASES WHICH COPURIFY IN PARTIALLY PURIFIED PREPARATIONS OF THE INSULIN RECEPTOR

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Abstract

HEPATIC INSULIN RECEPTOR TYROSINE KINASE ACTIVITY IN DIABETES: MODULATION BY ASSORTED ADENOSINE TRIPHOSPHATASES/PHOSPHATASES WHICH COPURIFY IN PARTIALLY PURIFIED PREPARATIONS OF THE INSULIN RECEPTOR. Darrick J. Alaimo, Arthur I. Salhanick, and John M. Amatruda. Endocrine-Metabolism Unit, Department of Internal Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York. (Sponsored by Robert Sherwin, Department of Internal Medicine, Yale University School of Medicine).

We evaluated the assay conditions for quantifying insulin receptor tyrosine kinase activation by insulin toward poly Glu/Tyr(4:1) in hepatic plasma membrane and microsomal wheat-germ agglutinin eluates prepared from normal and streptozotocin diabetic rats. Optimal insulin receptor tyrosine kinase conditions were found to include HEPES buffer, the absence of cytosine triphosphate, and the presence of sodium orthovanadate (1 mM) and para-nitrophenylphosphate (10 mM). Using Lineweaver-Burke analysis, the maximum insulin receptor tyrosine kinase activity was demonstrated to be reduced by 43% (6.37 and 3.64 pmol ^{32}P incorporated/ fmol insulin binding for control and diabetic preparations, respectively) in plasma membranes and by 42% (6.80 and 3.97 pmol ^{32}P incorporated/fmol insulin binding for control and diabetic groups, respectively) in microsomes from diabetic rats. Quantitation of ^{32}P-orthophosphate generation during incubations with [$\gamma^{32}P$]ATP revealed the presence of ATPase/phosphatase activity that copurified with insulin receptors from wheat-germ agglutinin

columns. This activity was present in preparations from plasma membranes and microsomes and had a vanadate-insensitive component in plasma membrane insulin receptor preparations. The decreased tyrosine kinase activity found in preparations from diabetic rats cannot be explained by this ATPase/phosphatase activity which was found to be lower (35-48%) in diabetic preparations.

The results provide the first evidence that the insulin resistance of nonketotic diabetes mellitus is associated with a reduction in hepatic plasma membrane insulin receptor tyrosine kinase and describe the presence of a copurifying ATPase/phosphatase activity. The mechanisms underlying the decrease in insulin receptor kinase in diabetes and the possible physiologic role for this copurifying ATPase/phosphatase activity require further investigation.

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Introduction

The insulin receptor is a transmembrane glycoprotein composed of two α-subunits and two β-subunits that are held together by disulfide bonds (reviewed in 1). The α-subunits are highly glycosylated, extramembrane peptides that bind insulin, while the β-subunits are glycosylated, transmembrane peptides that have intrinsic tyrosine kinase activity (1). The cloning of the insulin receptor gene has led to further definition of the structure of the insulin receptor. The β-subunit has been found to contain a tyrosine kinase domain that has a high degree of homology to other known tyrosine kinases such as the *src* oncogene, the epidermal growth factor (EGF) receptor, and the platelet-derived growth factor (PDGF) receptor (reviewed in 2,3). The β-subunit also has an adenosine triphosphate (ATP) binding region that appears to be necessary for tyrosine kinase activity (4,5).

The insulin receptor is responsible for mediating all of the biological effects of insulin. Short-term effects occur within minutes and include stimulation of nutrient uptake, glycolysis, glycogenesis, lipogenesis, and inhibition of lipolysis and gluconeogenesis (reviewed in 5). Long term effects occur over hours and include stimulation of cell growth, DNA synthesis, and protein synthesis (5). Certain effects of insulin will predominate in the cells of different organ systems (reviewed in 7). Liver and muscle cells are highly responsive to insulin's effects on gluconeogenesis, glycogenesis, and nutrient uptake, while adipocytes are more responsive to insulin's effects on lipolysis and lipogenesis.

The first step in insulin's action on a target cell is its binding to the α-subunit of the insulin receptor (1). The signal is transduced by an unknown mechanism, possibly by receptor clustering, that activates the

tyrosine kinase domain of the β-subunit (7). The β-subunit autophosphorylates to a tri-phosphorylated form in the tyrosine-1150 domain which is required for the tyrosine kinase to phosphorylate endogenous substrates (2,9,10). Although the phosphorylation of numerous endogenous uncharacterized proteins has been described (6,7,11,12,13), their relation to intracellular signaling and insulin's actions remains to be elucidated.

By unknown means, intracellular signals activate a variety of mechanisms which mediate the effects of insulin. For example, enhancement of glucose transport is mediated by translocation of a glucose transporter protein (glut-4) to the plasma membrane (PM) from intracellular storage vesicles (6,14). Metabolic effects of insulin could be the result of altering the activity of various kinases and phosphatases that are involved in controlling the activity of regulatory enzymes of metabolism (6). Another example of an intracellular effect of insulin is its effect on cell growth which occurs via alterations in the rates of DNA replication and transcription (6,7).

Many theories exist regarding the mechanisms by which insulin binding results in intracellular signaling. Some theories revolve around G-proteins (3,4). G-proteins are a group of intramembrane proteins that are involved in coupling certain receptors to their second messenger systems (i.e. β-adrenergic receptors coupled to adenylate cyclase via the G-protein, G_s). Although there are a number of different types of G-proteins, they all require the hydrolysis of guanosine triphosphate (GTP) for signal transduction to occur (4). Houslay (3) has suggested that a unique G-protein (G_{ins}) is involved in insulin-stimulated inhibition of adenylate cyclase because GTP is required. He also has argued for G_{ins} involvement in insulin activated cyclic adenosine monophosphate (cAMP)

phosphodiesterase (PDE) which requires GTP and can also be activated by Cholera toxin, an activator of G-proteins. The insulin receptor tyrosine kinase can phosphorylate G-protein subunits *in vitro*, and this ability could be a mechanism of interaction (3). Further evidence has come from a study (15) using streptozotocin-induced diabetic rats which showed that diabetic liver tissue had a loss in both amount and activity of G_i (a different type of G-protein) that was reversed by insulin administration. These results suggested that insulin regulates the expression of G_i in the liver. Because G_i mediates inhibition of adenylate cyclase and may couple receptors to potassium channels, decreased expression of G_i may be responsible in part for some of the physiologic changes found in diabetes (15).

Some evidence has accumulated for a role for the calcium-calmodulin second messenger system in the intracellular mechanism of action of insulin. In this second messenger system, an activated receptor system increases intracellular calcium concentration. Calcium then binds to the protein calmodulin creating a complex which activates a number of different calmodulin-sensitive enzymes (6). Calmodulin is phosphorylated at a tyrosine residue in the calcium binding region by the activated insulin receptor tyrosine kinase which could alter the activity of calmodulin (6). Insulin enhances calmodulin binding to adipocyte plasma membranes which may be related to the insulin-induced inhibition of a calmodulin-sensitive adenosine triphosphatase (ATPase) found in plasma membranes (2). There is evidence for calcium and calmodulin binding sites on the β-subunit of the insulin receptor, and these sites may be involved in regulation of insulin receptor autophosphorylation (2).

Internalization of insulin bound to its receptor also seems to be important in mediating the actions of insulin on various cellular processes.

Internalization occurs either by the clathrin coated pit pathway (10%) or by direct internalization via the non-coated pit pathway, and there are binding sites for insulin on most organelles (2,7). Inhibition of insulin receptor internalization and processing has been shown to inhibit a number of effects of insulin including stimulation of glucose transport, regulation of glycogen metabolism, and effects on RNA processing (2). Insulin and its receptor have been localized in nuclear membranes where insulin has been shown to activate a RNA-dependent nucleoside triphosphatase that provides energy to transport large molecules through nucleopores (2). Insulin may regulate messenger RNA efflux from the nucleus by this mechanism and thus influence protein synthesis (2). However, by using a mutated receptor, McClain has shown that the noninternalizing insulin receptor is fully capable of activating glycogen synthetase and stimulating mitosis in growth-arrested cells (16).

Other researchers have concentrated on the phosphatidylinositol second messenger pathway while studying the intracellular mechanism of action of insulin. In this second messenger system, an activated receptor system stimulates a phospholipase which cleaves phosphatidylinositol, an intramembrane phosphoglycolipid, to form inositol triphosphate and diacylglycerol (DAG). Inositol triphosphate stimulates the release of calcium from intracellular stores, while DAG activates Protein Kinase-C (PK-C) (6). The insulin receptor tyrosine kinase has been shown to stimulate phosphatidylinositol kinase, an enzyme involved in the synthesis of phosphatidylinositol, via phosphorylation (6,11), but no changes in inositol triphosphate or phosphatidylinositol levels have been found (6). Insulin has been shown to stimulate DAG synthesis (2,14), but changes in turnover have not been observed (2). Some studies have shown that insulin

has no effect on PK-C activity (2), while a recent study by Cherqui *et al.* (14) has provided evidence for myristoyl-DAG and PK-C involvement in insulin-stimulated glucose uptake. Their study showed that cells transfected with insulin receptor genes that were mutated at tyrosine residues 1162 and 1163 (major sites of autophosphorylation) had decreased tyrosine kinase activity and 2-deoxyglucose uptake. In addition, the concentration-dose response curves for insulin-stimulated myristoyl-DAG production and 2-deoxy-glucose uptake were superimposable in both normal and mutant cells. They also showed that insulin-stimulated phosphorylation of a 40 kDa protein (a known substrate for PK-C) was diminished in the mutant cells. However, direct stimulation of PK-C could stimulate phosphorylation of the 40 kDa protein and uptake of 2-deoxyglucose in mutant cells to near control levels. Thus, tyrosines 1162 and 1163 may control glucose uptake, at least in part, via myristoyl-DAG production and PK-C activation.

Related to the phosphatidylinositol system are studies that have concentrated on inositol phosphate glycans as mediators of insulin action. Insulin has been shown to stimulate (possibly via a G-protein) a specific phospholipase-C that liberates inositol phosphate glycans from PM phosphoglycolipids (2,6,7,11). Phospholipase-C may also release DAG with inositol phosphate glycans (6). Inositol phosphate glycans have been shown to inhibit adenylate cyclase and to stimulate cAMP PDE and thus may be involved in regulation of cAMP levels by insulin (6). These mediators are also able to stimulate liver pyruvate dehydrogenase activity (6). Thus, inositol phosphate glycans may be involved in some of insulin's actions. However, some investigators have been unable to confirm the existence of these mediators (6).

Although it is evident from the above discussion that multiple signaling pathways are involved in insulin action, increasing evidence suggests that the tyrosine kinase activity of the insulin receptor is involved in signal transmission. Insulin mimetic agents, such as concanavalin A, have been shown to increase insulin receptor tyrosine kinase activity (1). Agents that inhibit the insulin receptor tyrosine kinase have also been useful. Catecholamines and phorbol esters were shown to inhibit insulin-stimulated autophosphorylation, tyrosine kinase activity, and glucose transport in isolated human adipocytes (17). Synthetic competitive inhibitors of tyrosine kinases have been shown to inhibit insulin-stimulated antilipolysis and lipogenesis (18).

Studies with antireceptor antibodies have been more equivocal. Antibodies against the tyrosine kinase domain of the β-subunit inhibit some of insulin's actions (6), while a different antibody directed against the β-subunit has been shown to enhance insulin receptor tyrosine kinase activity and to stimulate 2-deoxyglucose transport, lipogenesis, and glycogen synthesis in isolated cells in a manner that was not additive to the effect of insulin (19). Other antibodies against the α-subunit have been shown to stimulate amino acid (20) and glucose (21) uptake in isolated cells without stimulating insulin receptor autophosphorylation or tyrosine kinase activity (20,21). These studies argued against an obligatory role for the insulin receptor tyrosine kinase in the transmembrane signaling to mediate all of insulin's actions. However, a study (4) using site-directed mutagenesis showed that the replacement of lysine residue 1030 in the putative ATP-binding domain of the insulin receptor abolished insulin-stimulated autophosphorylation, tyrosine kinase activity, and

2-deoxyglucose uptake. Interestingly, this mutation also prevented the stimulation of 2-deoxyglucose uptake by various antireceptor antibodies that had been previously shown to stimulate glucose uptake independent of autophosphorylation or tyrosine kinase activity.

Further studies with site-directed mutations of the insulin receptor have also suggested that the insulin receptor kinase is involved in mediating many but not all of insulin's actions. Studies using insulin receptors mutated in the ATP-binding domain and thus deficient in kinase activity have shown that the mutated cells also have impaired insulin-dependent 2-deoxyglucose uptake (4,5), S6 kinase activity, endogenous substrate phosphorylation, glycogen synthesis, and DNA synthesis (5). Site-directed mutation of tyrosine 960 did not affect autophosphorylation or tyrosine kinase activity toward exogenous substrates but did inhibit insulin-stimulated glycogen synthesis, amino acid uptake, DNA synthesis, and phosphorylation of an endogenous substrate (pp 185) (22). This study suggested that autophosphorylation was not sufficient to mediate some actions of insulin and that tyrosine phosphorylation of cellular substrates (which may involve tyrosine 960) was needed for signal transduction (22). As discussed above, site-directed mutations at major sites of autophosphorylation (tyrosines 1162 and 1163) result in diminshed tyrosine kinase activity, but the decrease in 2-deoxyglucose uptake may be more related to alterations in autophosphorylation, DAG production, and PK-C activity (14). Furthermore, one study with insulin receptors mutated at the ATP-binding domain showed that insulin-dependent stimulation of pyruvate dehydrogenase bypasses the insulin receptor kinase (23).

Many genetic studies of patients with various clinical syndromes of insulin resistance, including non-insulin-dependent diabetes mellitus

(NIDDM), have added evidence that the insulin receptor kinase is involved in signal transmission of insulin's actions. A number of studies on patients with Type A extreme insulin resistance have shown that insulin receptors from these patients have diminished autophosphorylation and kinase activity but normal binding *in vitro* (12,24-28) and are often mutated in the tyrosine kinase domain (12,25-28). Studies with these mutant receptors in isolated cells have shown that some mutations result in the inhibition of insulin-dependent phosphorylation of exogenous substrates (pp 185) (12,27), 2-deoxyglucose uptake (28), DNA synthesis (12), or gene expression (12). Yet, other mutations have no effect on insulin-stimulated 2-deoxyglucose uptake and glycogen synthesis (12). Thus, these and the above mentioned studies suggest that the insulin receptor kinase is involved in transmembrane signaling for many but not all of insulin's actions and that a divergence of insulin signaling pathways seems to occur at the level of the insulin receptor.

Many investigators have concentrated on quantitating insulin receptor autophosphorylation and kinase activity in an attempt to gain insight into the mechanisms responsible for various insulin resistant states, including NIDDM. Some studies have concentrated on insulin resistant states induced by obesity (13,29,30), fasting (31-33), hyperinsulinemia (34), or catecholamines and phorbol esters (17), to examine the effects on insulin receptor function, while others have focused on the insulin resistance of NIDDM (10,13,30,33,35-51). Researchers have found decreases (10,13,17, 30,31,34,36,37,39-43,45-50), increases (29,30,33,51), or no change (29,32,35,38, 44,49) in autophosphorylation (10,13,17,29-33,36,38,39,41-44,46,48,49,51) and tyrosine kinase (10,13,17,29-32,34-43,45-51) activities in insulin receptors exposed to the above mentioned states of insulin resistance. Tissues from

either insulin resistant humans (10,13,36,37,40, 45,47,50) or animals (17, 29-35,38,39,41-44,46,48,49,51) have been used in the studies. A majority of the above studies have shown that decreases in insulin receptor autophosphorylation and/or tyrosine kinase activity are present in insulin resistant subjects. These observations suggest that a defect(s) at the level of signal transmission is responsible for insulin resistance. However, as will be discussed below, a number of differences in models and methodology may make some studies less physiologically relevant.

While the transmembrane signaling mechanisms involved in insulin action have been studied intensely, only recently have investigations focused on the mechanism(s) by which the insulin receptor is deactivated. Unlike many other receptors, the insulin receptor tyrosine kinase remains active even after insulin dissociates from its binding site (11,52). The kinase can be inactivated by phosphorylation of serine residues in the β-subunit (6,11). Phorbol esters and cAMP have been shown to stimulate serine phosphorylation of the insulin receptor thus inhibiting the receptor kinase (6,11). However, some studies have suggested that this mechanism of receptor deactivation may be a mechanism of regulating insulin receptor activity by other hormones (i.e. glucagon, catecholamines) rather than a mechanism of terminating insulin's signal (6,17).

Many investigators have concentrated instead on the dephosphorylation of autophosphorylation sites by phosphotyrosine protein phosphatases as a mechanism of insulin receptor deactivation. A number of phosphotyrosine phosphatases have been isolated that have activity against the autophosphorylated insulin receptor or similar phosphorylated peptides (9,52-59), and some phosphatases can be stimulated by insulin (58,60,61). In addition, other phosphotyrosine phosphatases have been isolated that

possess activity against the phosphotyrosine forms of known exogenous substrates of the insulin receptor tyrosine kinase such as histones (62,63), casein (64,65), and poly Glu/Tyr(4:1) (54,66,67). Yet, no phosphatase activity is intrinsic to the insulin receptor (56). Thus, phosphotyrosine phosphatases may be involved in terminating insulin signal transduction at different levels of the signaling pathway.

Despite the growing evidence supporting the importance of phosphotyrosine phosphatases, only a few studies have addressed their role in the hepatic insulin resistance of diabetes. The results of these studies are not consistent, with reports of a decrease (55), increase (55), or no change (54) in phosphatase activity of microsomal (54,55) or PM (54) preparations from diabetic rats. In these studies, phosphatase activity was measured with an artificial ^{32}P-labelled substrate which may lack the sensitivity to highly-specific phosphatases (e.g. phosphopeptide 1142-1153) (55) or may not be representative of protein sequences (e.g. ^{32}P-RCM-lysozyme) (54) of the insulin receptor. The role of phosphotyrosine phosphatases in the pathogenesis of the insulin resistance of diabetes remains to be elucidated.

Previously, this laboratory has reported that autophosphorylation of the hepatic insulin receptor of streptozotocin-diabetic rats (a model of NIDDM in humans) was unaltered in partially-purified preparations extracted from PM (44). This study was initiated because previous studies (33,43) examined hepatic insulin receptor preparations made from microsomal membranes which may not be physiologically relevant when considering insulin action. Similarly, more recent studies (29,30,35,38,39,41,45,48,49) examining insulin receptor autophosphorylation and tyrosine kinase activity in diabetes have also used preparations not of PM origin. The relevance of these investigations is also questionable, especially because a

previous study from this laboratory (68) revealed that the contribution of PM is only about 20 percent of the total cellular insulin binding in the hepatocyte. The present work establishes assay conditions and extends the previous study (44) by evaluating hepatic insulin receptor kinase activity in both PM and microsomal preparations from normal and streptozotocin diabetic rats. In addition it describes the presence of copurifying ATPases and phosphatase activities in these extracts

The type of buffer to be used in the study needs to be assessed during the evaluation of assay conditions. The majority of the previously mentioned studies have used either Tris (33,34,37,45,59,63,66,67) or HEPES (10,17,29,30, 32,36,39-44,46-53,55-57) as buffers. However, there is some evidence that Tris-containing buffers may reduce insulin binding capacity (69,70), but the ability of Tris to perturb insulin receptor kinase activity has not been previously evaluated.

Another variable in previous studies that needs to be evaluated is the use of cytosine triphosphate (CTP). A number of studies (31,35,36,38,40, 47,50,71-74) have used CTP as an inhibitor of adenosine triphosphatases (ATPases) that may copurify with the insulin receptor and diminish the apparent receptor kinase activity by limiting its substrate, ATP. CTP is used because it does not compete with ATP at the insulin receptor kinase (1) but is a good substrate for a number of broad specificity ATPases isolated from hepatocyte membranes (75,76). When the effect of CTP on ATPase activity has been evaluated, CTP has been effective in inhibiting ATPases in most studies (36,38,50,71,74), but some investigators have found no effect (31) or inconsistent effects (47) of CTP on ATPase activity. A 10:1 ratio (or greater) of CTP:ATP concentrations should be sufficient for inhibition of ATPases (76). Ratios of 10 or 20 are used in the majority of studies

(31,36,40,50,71,72). In the present study, 2 mM CTP is used to give a [CTP/ATP] ratio of 20:1.

The use of vanadate in previous studies has not been consistent and warrants evaluation in the present study. Vanadate is a potent inhibitor of many, but not all, phosphotyrosine phosphatases and ATPases (77,78) that may copurify with insulin receptors purified on wheat germ agglutinin columns (32,79,80). The majority of studies that have used vanadate and evaluated its effect on phosphatase and/or ATPase activities have found it to be effective (31,38,49,50,71,74), while others have found no inhibitory effect of vanadate (33,36,47,79). Most investigators who have found vanadate to be effective have used concentrations of 0.1 to 1.0 mM (31,38,49,50,74) which is consistent with the fact that most phosphotyrosine phosphatases that have been tested from rat liver are inhibited by 0.1 and 1.0 mM vanadate concentrations (52-54,56,59). In the present work, 0.1 and 1.0 mM concentrations are chosen to be evaluated.

The effectiveness of vanadate in inhibiting ATPases/phosphatases also requires assessment. This ATPase/phosphatase activity can be detected by measurement of ^{32}P-orthophosphate ($^{32}P_i$) generation during incubations of insulin receptor preparations with [γ^{32}P] ATP. The molybdate technique (81), which has been used in previous studies (38,43,74,82), is being used in the present study because it allows the measurement of a large number of samples taken from very small sample volumes. The data from the present study show the presence of ATPase/phosphatase activity that copurifies with the insulin receptor through lectin affinity chromatography.

In order to fully evaluate the activity of the insulin receptor tyrosine kinase in these preparations it is necessary to assess activity when substrate (ATP) conditions are not limiting. This evaluation can be

accomplished by varying the ATP concentration and doing Lineweaver-Burke analysis to determine the maximum velocity (V_{max}) and Michaelis constant (K_m) for the insulin receptor kinase (34). The data reveals that microsomal and PM insulin receptors from diabetic rat livers have decreased kinase activity compared to control rat hepatic insulin receptors. Since the previous study from this laboratory (44) was performed at lower ATP concentrations and in the absence of vanadate, the ATPase/ phosphatase activity detected in this work may explain the earlier finding of normal kinase activity in insulin receptors prepared from PM of diabetic rats. The results provide the first evidence that the insulin resistance of nonketotic diabetes mellitus is associated with a reduction in insulin receptor tyrosine kinase activity in hepatic PM.

Methods

Reagents. Streptozotocin was a gift from The Upjohn Company (Kalamazoo, MI); aprotinin, phenylmethylsulfonyl fluoride, poly Glu/Tyr (4:1), bovine serum albumin (fraction V), sodium orthovanadate, and unlabelled nucleotides were obtained from Sigma (St. Louis, MO); wheat-germ agglutinin-agarose (WGA) was from E-Y Laboratories (San Mateo, CA); crystalline porcine insulin was a gift from Eli Lilly and Company (Indianapolis, IN); [γ^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Corporation (Chicago, IL); carrier-free [^{125}I]Na was from New England Nuclear (Boston, MA); Dextran T-500 was from Pharmacia-LKB (Piscataway, NJ). All other chemicals were reagent grade.

Animals. Male-Sprague Dawley rats, approximately 2 months old and fed *ad libitum* were used for all studies. Non-ketotic diabetes was induced by the injection of streptozotocin (85mg/kg) via femoral vein as described previously (83). Rats were used 2 to 4 weeks after injection and had elevated serum glucose (>450 mg/dl) and low serum insulin (<8 μU/ml).

PM Isolation and Lectin-Purification of the Hepatic Insulin Receptor. Purified PM were isolated from 20 g of rat liver from normal and diabetic rats as previously described (44,68). Marker enzyme profiles of these preparations have been previously reported (68). PM were extracted by 2-phase polymer method as previously described (68,84). Aqueous polymer phases were prepared by mixing 30%(w/w) Polyethylene Glycol-6000 (PEG) in H_2O with 20%(w/w) Dextran-500 in H_2O and 0.22 M phosphate buffer (pH 6.5), allowing to stand at 4°C for 48 hours, and then collecting the top and

bottom phases separately. Liver homogenization was performed at 4°C in the presence of 1 mM phenylmethylsulfonyl fluoride and 1.0 TIU/ml aprotinin. The homogenate was centrifuged at 1200xg for 30 minutes, and the pellet was saved and washed with 2 additional 1000xg centrifugations for 15 minutes each. The pellets were saved, suspended in top and bottom phases, and centrifuged in a swinging bucket rotor at 1100xg for 15 minutes. PM were removed from the interface between the polymer phases using a pasteur pipet, resuspended in top and bottom phases, and centrifuged at 1100xg for 15 minutes. This collection and separation of PM was repeated once more with the polymers, and then PM were washed in 50 mM Tris (pH 7.5) to dispel the polymers and spun at 2400xg for 10 minutes. This Tris wash was repeated once, and then the pellets were then resuspended in HEPES buffer, homogenized and stored at -70°C. PM insulin receptors were extracted as previously described (68,84) except that the final Triton X-100 concentration was increased from 1 to 2%(v/v). After clarification of the detergent extract by centrifugation at 100,000xg for 60 minutes, the supernatant was diluted to 0.5%(v/v) Triton X-100 and recycled three times through a WGA affinity column (44). After washing the column with 50 mM HEPES buffer, pH 7.6, containing 150 mM NaCl, 10 mM $MgSO_4$, 0.02%(w/v) sodium azide, and 0.1%(v/v) Triton X-100, elution of the insulin receptor was achieved by the inclusion of 300 mM N-acetyl-D-glucosamine in the above buffer. The protein content of column eluates was determined by the dye-binding (Coomassie Blue) method of Bradford (85) (Bio Rad Protein Assay) using bovine gamma globulin as the standard. Aliquots were frozen at -70°C. The protein concentration of pooled fractions was generally 0.8 to 1.4 mg/ml.

Preparation of Microsomes. Microsomal membrane preparations were prepared at 4°C from 20 grams of liver following homogenization in 50 mM HEPES buffer, pH 7.6, containing 250 mM sucrose, 1 mM phenylmethyl-sulfonyl fluoride and 1 TIU/ml aprotinin. The homogenate was centrifuged at 10,000xg for 20 minutes, the supernatant removed, and centrifuged at 100,000xg for 60 minutes. The pellet (microsomes) was saved, washed with an additional 100,000xg centrifugation, and partially-purified on WGA columns following solubilization in Triton X-100 as described above.

Insulin Binding of WGA-Column Eluates. ^{125}I-insulin was prepared by the Chloramine-T method as described by Cuatrecases (82). Prior to use, the iodinated insulin was purified on a Bio-Gel P-30 column. Aliquots (5 to 15 μg of protein) of WGA column eluates were incubated with ^{125}I-insulin (1 nM) in the presence and absence of 1 μM unlabelled insulin for 15 to 120 minutes at 24°C in phosphorylation buffer (see below), unless otherwise noted. The incubation was terminated by placing on ice and adding bovine gamma globulin and 10% PEG as previously described (68). Samples were then centrifuged for 1 minute in a Beckman microfuge at 10,000xg. Pellets were then washed with 10% PEG and counted in a gamma-counter. Samples were run in quadruplicate, and data expressed in fmol insulin bound/mg protein ± standard deviation. For kinase and other assays, equal binding for diabetic and control preparations was determined by varying receptor protein concentrations at the same time of incubation, and then the appropriate preparation was diluted with phosphorylation buffer. Preliminary studies established that recovery of receptors with WGA chromatography was comparable in liver extracts from control and diabetic rats.

Insulin Receptor Tyrosine Kinase Activity. Aliquots of lectin-purified PM and microsomal insulin receptor preparations containing equal binding activity (bound/total ranged from 10 to 15%) from normal and diabetic rats were incubated at 24°C for 90 to 120 minutes in the absence and presence of 100 nM insulin in a buffer solution (total volume of 30 µl) modified from Zick *et al.* (82). Except where noted, the phosphorylation buffer consisted of 50 mM HEPES buffer, pH 7.6, 50 mM NaCl, 20 mM $MgCl_2$, 2 mM $Mn(CH_3COO^-)_2$, 0.02%(w/v) bovine serum albumin, and 0.1%(v/v) Triton X-100. Phosphorylation was initiated by the addition of a 10 µl solution containing [γ^{32}P]ATP (final concentration 100 µM), sodium orthovanadate (final concentration 0, 0.1, or 1 mM), p-nitro-phenylphosphate (pNPP) (final concentration 0 or 10 mM), and CTP (final concentration 0 or 2 mM). After a 10 minute incubation at 24°C, 10 µl of phosphorylation buffer containing poly Glu/Tyr (4:1) (final concentration was 1 mg/ml) were added (total volume of 50 µl), and the incubation continued for an additional 5 to 20 minutes. The reaction was terminated with one-half volume of a "stopping solution" (82) consisting of 50 mM HEPES buffer, pH 7.4, 40 mM NaH_2PO_4, 20 mM EDTA-Na, 200 mM NaF, 40 mM sodium pyrophosphate, 40 mM ATP, 20 mM pNPP, 5 mM sodium orthovanadate, and 0.4% Triton. Aliquots were precipitated with tricholoracetic acid on Whatman 3MM filter papers as described by Zick *et al.* (72). Filter papers were dried then placed in cold 10% trichloroacetic acid (TCA) for 90 minutes. Filter papers were then rinsed, and cold 10% TCA was replaced for 60 minutes. A third 10% TCA rinse of 15 minutes was performed, and then filter papers were washed in 95% ETOH for 10 minutes. Filter papers were rinsed with diethylether (reagent), dried, and then counted by liquid scintillation.

Insulin dose response for tyrosine kinase activity was assessed by the above procedure, but the insulin concentration was varied from 0 to 10^{-7} M. Background subtractions were made by determining radioactivity on filters prepared from incubations performed in the absence of insulin receptor and/or poly Glu/Tyr(4:1). Kinase activity was expressed in pmol ^{32}P incorporated/fmol insulin-binding over 10 minutes of kinase incubation. Samples were run in triplicate or quadruplicate.

Evaluation of Trichloroacetic Acid Wash Procedure. The TCA wash procedure was used to precipitate the ^{32}P-Glu-Tyr(4:1), as described above. The efficiency of the washes was evaluated in the following manner. Control microsomal insulin receptors were incubated with [$\gamma^{32}P$]ATP for 10 minutes and then quenched with the "stopping solution" as described above. Aliquots were spotted on filter papers and placed in 10% TCA. To assess the stickiness of [$\gamma^{32}P$]ATP, blank filter papers were also placed in the TCA. In wash procedure #1 washes were 90 minutes and then three 60 minute washes, while in wash procedure #2 washes were 90 minutes and then three 15 minutes washes. Blank and sample filter papers were withdrawn (in quadruplicate) and counted after each TCA wash. Data was expressed as pmol ^{32}P on filter paper/fmol of insulin bound.

Determination of Orthophosphate. ^{32}P-orthophosphate content was determined by the molybdate technique as described by Reinmann and Umfleet (81). Aliquots from incubations were applied to Whatman 3MM filter papers which were then placed in molybdate solution consisting of 1.5% TCA, 0.74% ammonium molybdate, and 7.4 mM triethylamine. Filter papers were swirled occasionally for 10 minutes. Molybdate solution was

then used to wash the filter papers for four more 10 minute periods. Filter papers dried overnight and then were counted in a beta counter. Data was expressed as either percent hydrolysis or pmol ^{32}P-orthophosphate formed/fmol insulin binding over 20 minutes of hydrolysis. Samples were run in quadruplicate.

Lineweaver-Burke Analysis. Insulin receptor tyrosine kinase activity was assayed as discussed above except that the concentration of [γ^{32}P]ATP was varied from 25 μM to 400 μM. Data was expressed as above but was plotted as a double reciprocal graph to allow Lineweaver-Burke analysis as described in (29). Linear regression was performed in order to determine maximum velocity (V_{max}) and Michaelis constant (K_m) for each set of data.

Statistics. Statistical analyses were performed by using the non-paired two-tailed Student's t Test.

Disclaimer. I performed all of the above methods with the exceptions of the preparation of ^{125}I-insulin by the Chloramine-T method and the determination of rat serum levels of glucose and insulin.

Results

Insulin Binding of WGA-Column Eluates. Partially-purified insulin receptors from PM and microsomes isolated from control and streptozotocin-diabetic rats were evaluated for insulin binding (tracer). As shown in Figure 1, insulin binding to hepatic microsomal insulin receptors from control and diabetic rats approached equilibrium between 60 and 90 minutes at 24°C which is similar to the results of other studies performed at the same temperature (42,43,46). As reported by others (30,39,41,43, 48,49), insulin binding was found to be higher (55-89%, 481±85 and 747±66 fmol insulin bound/mg protein for normal and diabetic preparations respectively at 120 minutes incubation, $p<0.05$) (mean ± standard deviation) in WGA extracts from diabetic rat livers (Figure 1). This result was in contrast with one study (35) which found decreased (by 50%) insulin binding in alloxan-diabetic rat preparations. Previous studies (35,39,49) have shown that these diabetes-induced changes in insulin binding are due to alterations in receptor number and not receptor affinity.

Insulin binding in PM insulin receptors from control and diabetic rats approached equilibrium in 60 to 90 minutes (Figure 2). This equilibrium time was similar to that observed with microsomal preparations at 24°C (Figure 1). Consistent with previous studies (38,44,45,51,68) but in contrast to the results with microsomes, no difference in insulin binding activity was found between control and diabetic PM insulin receptor preparations, (261±104 and 285±118 fmol insulin bound/mg protein for normal and diabetic preparations, respectively) (Figure 2). A previous

Figure 1. **^{125}I-INSULIN BINDING IN HEPATIC MICROSOMAL INSULIN RECEPTORS**

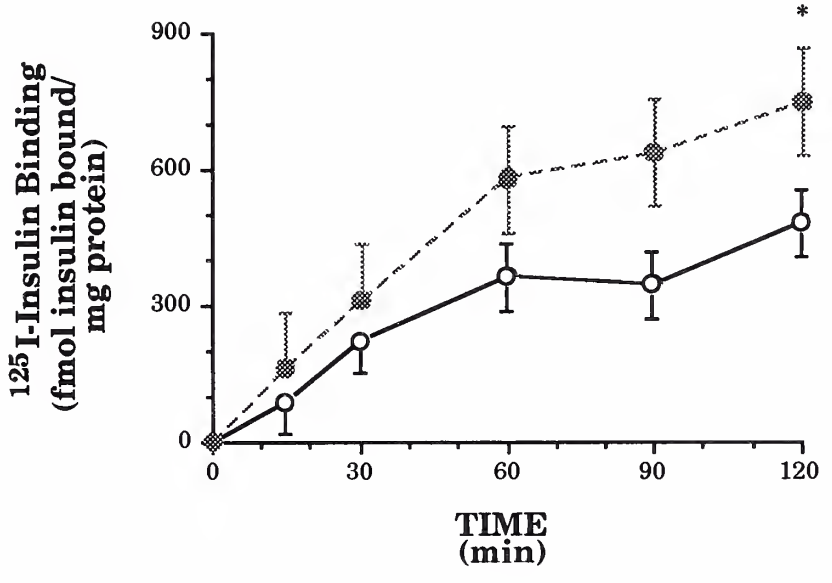
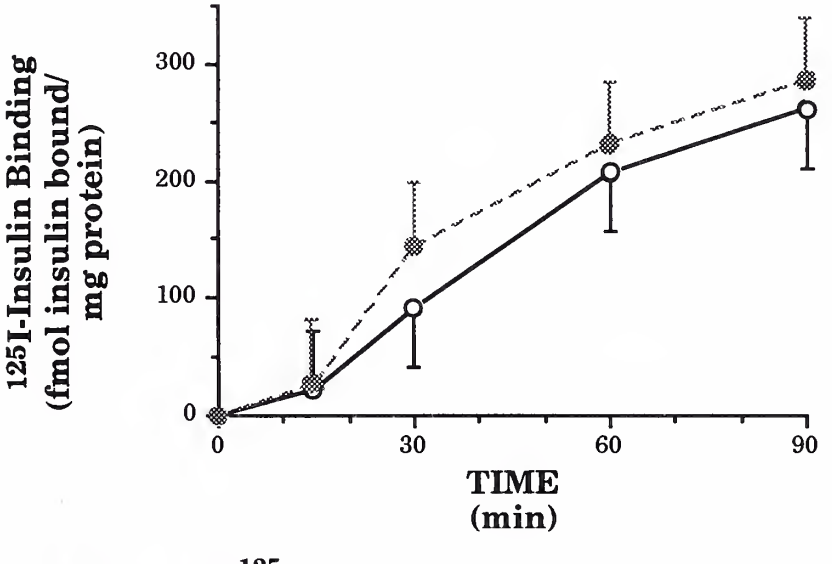


Figure 2. **^{125}I-INSULIN BINDING IN HEPATIC PM INSULIN RECEPTORS**



Figures 1,2. **^{125}I-insulin binding in hepatic insulin receptors. Aliquots of lectin-purified hepatic microsomal (Figure 1) and PM (Figure 2) insulin receptors from normal (open circles) and diabetic (solid circles) rats were incubated with ^{125}I-insulin (1nM) in the presence and absence of unlabelled insulin (1μM) for 15 to 120 minutes in phosphorylation buffer as described in Methods. The data represent the mean values from two to four experiments each performed in quadruplicate. Statistical comparisons between control and diabetic groups. * $p<0.05$.**

study (68) has also shown no difference in insulin binding affinity between control and diabetic PM insulin receptors.

Evaluation of Trichloroacetic Acid Wash Procedure. Figure 3 shows that the effectiveness of the washing of filter papers with TCA seemed to be more dependent on the number of washes rather than on the duration of each wash. Radioactivity on the filter papers with receptors reached a near steady-state by the third wash of each wash procedure (Figure 3). Presoaking the filter papers as described by Zick *et al.* (72) had no effect on results (data not shown). Blank filter papers showed that the degree of [γ^{32}P]ATP "stickiness" had also diminshed to a steady state by the third wash and was a small percentage of the microsomal receptor activity (14-18%) (Figure 3). For all subsequent experiments, a wash procedure of 90, 60, and 15 minute washes was employed.

Determination of Incubation Conditions for Insulin-Stimulated Tyrosine Kinase Assay. The effects of pNPP and vanadate on both insulin binding and tyrosine kinase activity were assessed in control and diabetic microsomal preparations initially equalized for binding in buffer lacking these agents. As shown in Figure 4, pNPP (10 mM) and vanadate (0.1 and 1.0 mM) decreased insulin binding in microsomal insulin receptors by 20 to 29%. The relationship of insulin binding between diabetic and control persisted in all three conditions. Because of this decrement in insulin binding with pNPP and vanadate, later experiments involving these inhibitors were conducted so that these inhibitors were added only with and after the addition of [γ^{32}P]ATP.

Figure 3. **EVALUATION OF TCA WASH PROCEDURE**

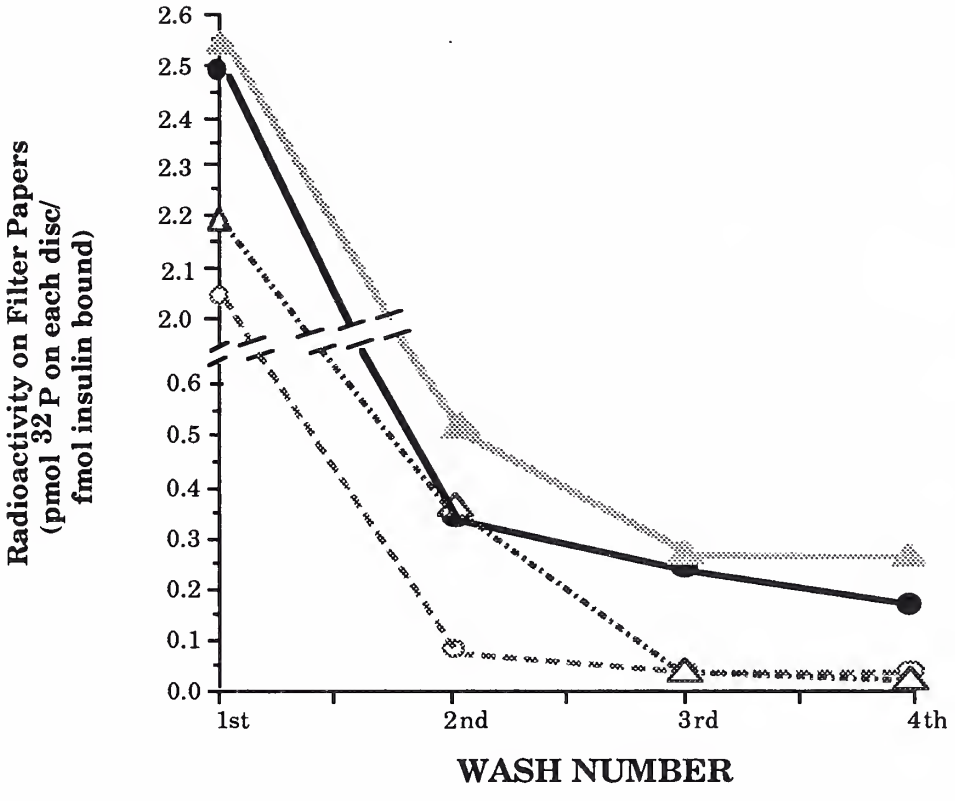


Figure 3. Evaluation of trichloroacetic acid wash procedure. As described in Methods, filter paper discs contained radioactivity from microsomal insulin receptors incubated with [γ^{32} P]ATP (solid circles and triangles) or from blank discs (open circles and triangles). Discs were washed with four successive washes according to procedure #1 (90 minutes then 60, 60, 60 minutes: circles) or procedure #2 (90 minutes then 15, 15, 15 minutes: triangles). Discs were removed in quadruplicate after each wash and counted. The data represent mean values from one experiment.

Figure 4. **EFFECT OF pNPP AND VANADATE ON INSULIN BINDING**

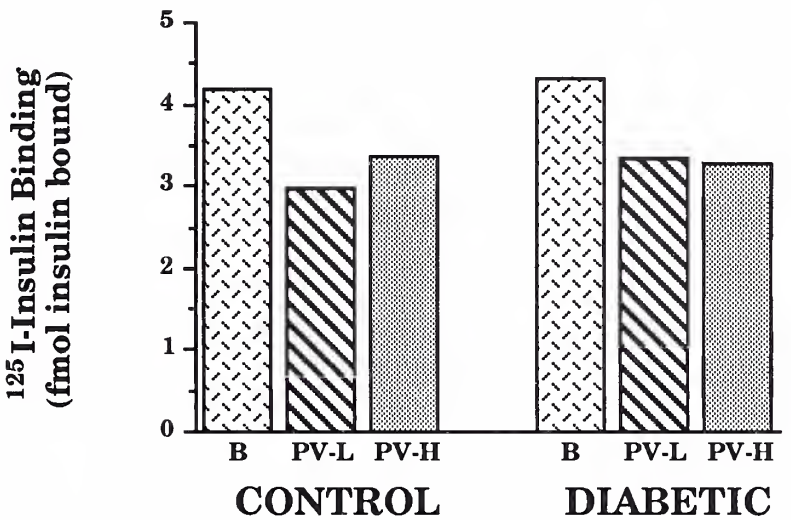


Figure 4. **Effect of pNPP and vanadate on insulin binding. Aliquots of hepatic microsomal insulin receptors equalized for binding were incubated in ^{125}I-insulin in the absence and presence of phosphorylation buffer containing 10 mM pNPP and 0.1 and 1.0 mM vanadate. (B=kinase buffer without inhibitors; PV-L, PV-H=kinase buffer with 10 mM pNPP and 0.1 or 1.0 mM vanadate, respectively.) Data represent mean values from one experiment performed in quadruplicate.**

Preliminary experiments without pNPP or vanadate showed minimal insulin-stimulated tyrosine kinase activity and a high degree of variability in both microsomal and PM preps. As shown in Figure 5 (incubation time with poly Glu/Tyr[4:1]=10 minutes), the addition of pNPP (10 mM) and vanadate (0.1 [PV-L] or 1.0 mM [PV-H]) to microsomal preparations enhanced insulin-stimulated tyrosine kinase activity in the control group (from 1.35±1.40 to 3.49±0.80 {+159%} or 3.60±1.14 {+167%} with PV-L or PV-H, respectively pmol ^{32}P incorporated/fmol insulin binding) and in the diabetic group (from 1.73±0.91 to 2.02±0.30 {+17%} or 2.86±0.85 {+65%} with the addition of PV-L or PV-H, respectively). The relationship between control and diabetic groups from microsomes was also altered with the addition of vanadate and pNPP. Without inhibitors the diabetic kinase activity was greater than the control (% Diabetic/Control [%D/C]= +28%), whereas with inhibitors the opposite was true [%D/C= -42% (PV-L), -21% (PV-H)].

As shown in Figure 6 (incubation time with poly Glu/Tyr[4:1]=10 minutes), the addition of pNPP and vanadate to PM insulin receptor preparations enhanced insulin-stimulated tyrosine kinase activity. In the control group, kinase activity increased from .09±.07 to 1.26±.56 or 1.45±.76 with PV-L or PV-H, respectively (pmol ^{32}P incorpo- rated/fmol insulin binding). Similarly, in the diabetic group kinase activity increased from .01±.01 to 1.84±.88 or 1.63±.84 with PV-L or PV-H, respectively (same units as above). The effect of pNPP and vanadate on the variability of the data was hard to assess because of the very low kinase activity without inhibitors. The large increase in kinase activity with vanadate suggested a high degree of contamination with ATPases/ phosphatases. The relationship between control and diabetic groups of PM origin was also

Figure 5. EFFECT OF pNPP AND VANADATE ON INSULIN-STIMULATED TYROSINE KINASE ACTIVITY IN MICROSOMES

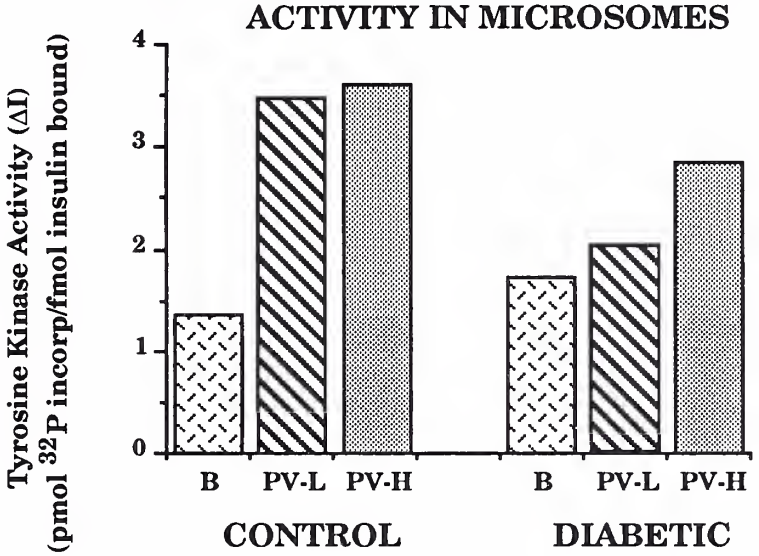
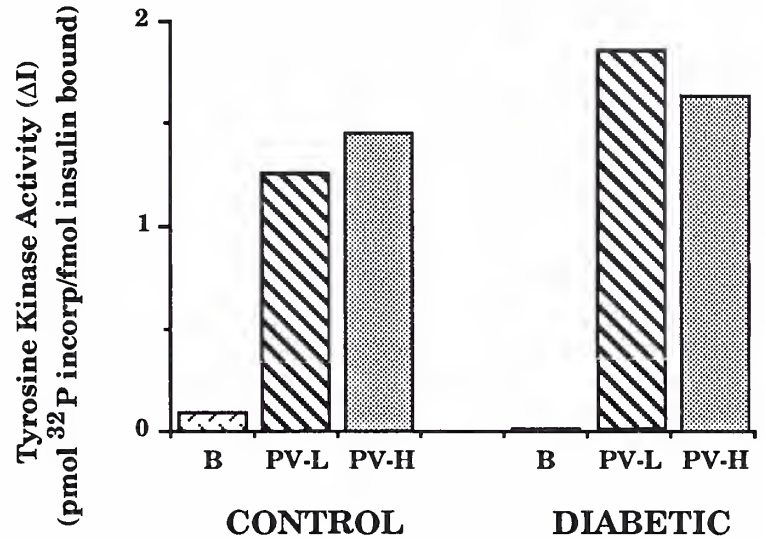


Figure 6. EFFECT OF pNPP AND VANADATE ON INSULIN-STIMULATED TYROSINE KINASE ACTIVITY IN PM



Figures 5,6. **Effect of pNPP and vanadate on insulin stimulated tyrosine kinase activity (ΔI) in microsomal (Figure 5) and PM (Figure 6) preparations. Aliquots of lectin-purified hepatic microsomal and PM insulin receptors from normal and diabetic rats were incubated in the presence of 100 nM insulin, 100μM [γ ^{32} P]ATP, and phosphorylation buffer with varying concentrations of pNPP and vanadate. (B=phosphorylation buffer without inhibitors; PV-L, PV-H= kinase buffer with 10 mM pNPP and 0.1 or 1.0 mM vanadate, respectively). Data represents mean values two to four experiments performed in quadruplicate. Insulin-stimulated tyrosine kinase activity (ΔI) was defined as kinase activity with insulin minus kinase activity without insulin. Incubation time with poly Glu/Tyr(4:1) was 10 minutes.**

altered with the addition of pNPP and vanadate. Without inhibitors both diabetic and control insulin receptors exhibited very little insulin-stimulated kinase activity, but the control had slightly more kinase activity than the diabetic prep. With inhibitors the diabetic kinase activity was greater than the control [%D/C= 1.84±.88/1.26±.56= +46%(PV-L); %D/C= 1.63±.84/1.45±.76= +12%(PV-H)], but there was a high degree of variability which precluded statistical comparisons. These increases in kinase activity in diabetic PM is in contrast to the previous study by this laboratory (44) in which insulin-sensitive insulin receptor autophosphory- lation was unchanged in the diabetic, but in the PV-H group the diabetic and control kinase activities were more similar than in the PV-L group.

The effect of Tris buffer on insulin-stimulated tyrosine kinase activity in microsomal insulin receptors was measured in the presence of 0.1 mM vanadate and 10 mM pNPP (incubation time with poly Glu/Tyr[4:1]=10 minutes). An initial experiment established insulin binding to be identical when comparing activity in HEPES or Tris and to have no effect in the insulin binding relationship of control and diabetic microsomal prepa-rations (data not shown). As shown in Table 1, Tris buffer reduced tyrosine kinase activity in the control preparation by 36% (7.94 and 5.08 pmol ^{32}P incorporated/fmol insulin binding for HEPES and Tris buffers, respectively) but had little effect on kinase activity in the diabetic preparation (4.00 and 4.13 for HEPES and Tris buffers, respectively in same units as above). The relationship of less kinase activity in the diabetic preparation remained intact although Tris decreased this difference. Because Tris buffer selectively reduced insulin receptor kinase activity in the control preparation, all subsequent experiments were conducted in HEPES buffer.

Table 1. **Effects of Tris and HEPES Buffers on Insulin-Stimulated Tyrosine Kinase Activity in Microsomes**

Tyrosine Kinase Activity (ΔI)
(pmol ^{32}P incorp/fmol insulin bound.)

BUFFER	CONTROL	DIABETIC	% D/C
Hepes	7.94	4.00	-50%
Tris	5.08	4.13	-19%
% change due to Tris	-36%	+3%	

Table 1. Effects of Tris and HEPES buffers on insulin-stimulated tyrosine kinase activity in microsomes. Aliquots of lectin-purified hepatic microsomal insulin receptors from control and diabetic rats were incubated in the presence of 100 nM insulin, poly Glu/Tyr(4:1)(incubation time=10 minutes), 100 μM ATP, 10 mM pNPP, 0.1 mM vanadate, and phosphorylation buffer made with either Tris or HEPES. The data represent the mean values from one experiment performed in quadruplicate.

Table 2. **Effect of CTP on Insulin-Stimulated Tyrosine Kinase Activity in Microsomes**

Tyrosine Kinase Activity (ΔI)
(pmol ^{32}P incorp/fmol insulin bound).

CTP	CONTROL	DIABETIC	%D/C
0 mM	3.12	1.75	-44%
2 mM	0.89	0.55	-38%
% change due to CTP	-71%	-69%	

Table 2. Effect of CTP on insulin-stimulated tyrosine kinase activity in microsomes. Aliquots of lectin-purified hepatic microsomal insulin receptors from control and diabetic rats were incubated in the presence of 100 nM insulin, poly Glu/Tyr(4:1)(incubation time=10 minutes), 100μM ATP, 10 mM pNPP, 0.1 mM vanadate, and CTP (0 or 2 mM). The data represent the mean values from one experiment performed in quadruplicate.

The effect of CTP on insulin-stimulated tyrosine kinase activity was investigated using hepatic microsomal insulin receptors isolated from control and diabetic rats. Insulin receptors were incubated in the presence of 0.1 mM vanadate and 10 mM pNPP (incubation time with poly Glu/Tyr[4:1]=10 minutes). Table 2 shows that the addition of CTP (2mM) resulted in a similar decrease (ca. 70%) in insulin-stimulated kinase activity in the control and diabetic preparations. This result is in contrast to previous studies in which CTP enhanced insulin receptor tyrosine kinase activity (36,38,50,71,74) or had no effect on tyrosine kinase activity (31). Although the relationship between diabetic and control kinase activities (%D/C= -44%) was preserved in the presence of CTP (%D/C with CTP= -38%), CTP was omitted in subsequent experiments because of the negative influence on both diabetic and control receptor activities.

Evaluation of the Time Course of Insulin Receptor Tyrosine Kinase Activation. Experiments were performed in the absence or presence of 1.0 mM vanadate and 10 mM pNPP (PV-H). Incubation time with poly Glu/Tyr(4:1) was varied from 5 to 20 minutes in order to evaluate the time course of microsomal and PM insulin receptor kinase activity isolated from control and diabetic rats. In microsomal hepatic insulin receptors without vanadate and pNPP, the control group kinase activity approaches equilibrium (5-10 minutes) before the diabetic group kinase activity (10-20 minutes) (Figure 7). However, when vanadate and pNPP were added to the reaction mixture, activation of both control and diabetic receptor kinases was nearly linear for up to 20 minutes. All other experiments with microsomes were incubated with poly Glu/Tyr(4:1) for 10 minutes in order to make linear measurements of tyrosine kinase activity.

Figure 7. **TIME COURSE OF INSULIN RECEPTOR TYROSINE KINASE ACTIVATION IN MICROSOMES**

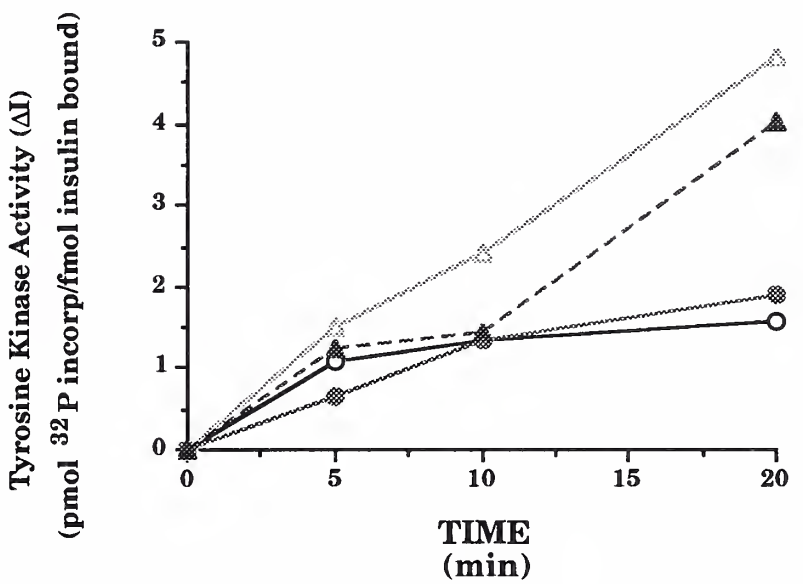


Figure 7. **Time course of insulin-stimulated tyrosine kinase activity in microsomal preparations. Aliquots of lectin-purified hepatic microsomal insulin receptors from normal (open circles and triangles) and diabetic (solid circles and triangles) rats were incubated in the presence of 100 nM insulin, 100 μM [γ^{32} P]ATP, and phosphorylation buffer without (circles) or with (triangles) pNPP (10 mM) and vanadate (1 mM). Incubation time with poly Glu/Tyr(4:1) was varied from 5 to 20 minutes. Data represent the mean from two experiments performed in quadruplicate.**

This incubation time was shorter in comparison to those used in previous studies conducted at 24°C (31,34,37,38,46). In one study conducted without vanadate, incubation time was 20 minutes (34), but those studies done in the presence of vanadate used incubations of 15 to 30 minutes (31,37,38,46). Interestingly, one study (30) that was conducted at 4°C with vanadate showed that microsomal insulin receptor tyrosine kinase activity was linear with respect to time for up to 120 minutes.

Diabetic microsomal receptor kinase activity was less than control activity at all time points to a similar degree (%D/C= -16% to -40%) when incubated with vanadate and pNPP. In their absence, control kinase activity was greater than diabetic activity at 5 minutes but had become less than diabetic activity at 20 minutes. These results thus suggest the presence of a greater degree of ATPase/phosphatase activity (causing [ATP] to become more limiting with time) in the control preparation.

In PM, the time course of insulin receptor tyrosine kinase activation could only be assessed in the presence of vanadate and pNPP because poor insulin responses were observed without inhibitors. As shown in Figure 8, kinase activation in both control and diabetic insulin receptors remained nearly linear with respect to time through 10 minutes of incubation. However, by 20 minutes both control and diabetic activities had plateaued. The former plateaued sooner and at a lower kinase activity which suggested that [ATP] was becoming limiting or dephosphorylation was occurring and to a larger degree in the control preparation. Consequently, an incubation time of 10 minutes was chosen for all other experiments with PM.

Evaluation of the Effect of Insulin Concentration on Insulin

Figure 8. **TIME COURSE OF INSULIN RECEPTOR TYROSINE KINASE ACTIVATION IN PM**

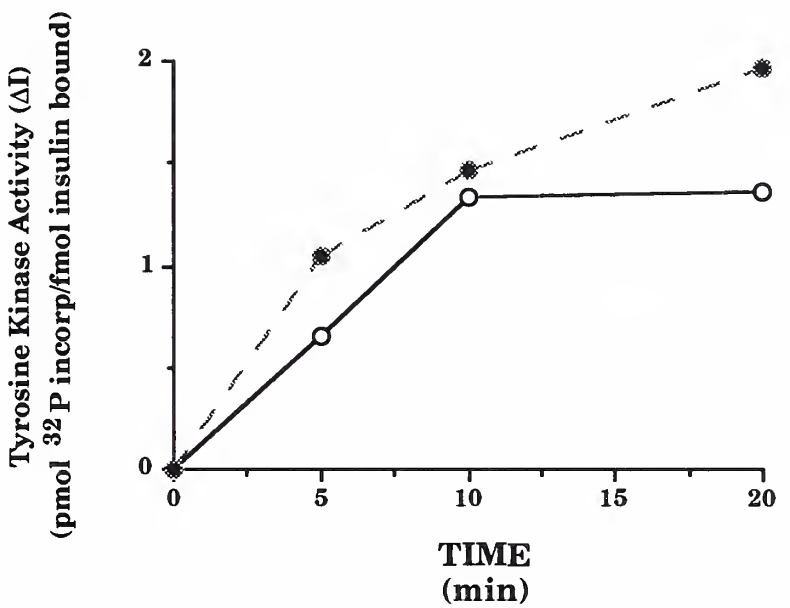


Figure 8. **Time course of insulin-stimulated tyrosine kinase activity in PM preparations. Aliquots of lectin-purified hepatic PM insulin receptors from normal (open circles) and diabetic (solid circles) rats were incubated in the presence of 100 nM insulin, 100 μM [γ^{32}P]ATP, and phosphorylation buffer with pNPP (10 mM) and vanadate (1 mM). Incubation time with poly Glu/Tyr(4:1) was varied from 5 to 20 minutes. Data represent the mean values from two experiments performed in quadruplicate.**

Receptor Tyrosine Kinase Activity. The effect of insulin concentration on tyrosine kinase activity was evaluated in both microsomal and PM insulin receptors in the presence of 0.1 mM vanadate and 10 mM pNPP as described in Methods. As shown in Figures 9 and 10, microsomal and PM insulin receptor kinases were activated at both physiologic (0.1 and 1.0 nM) and pharmacologic (10 and 100 nM) insulin concentrations. Physiologic and pharmacologic concentrations of insulin have previously been shown to stimulate the insulin receptor tyrosine kinase in intact hepatocytes (80).

Figure 9 shows that the dose response curves for control and diabetic microsomal receptor kinase activities were almost identical in shape. Diabetic and control kinase activities were similar at all insulin concentrations in this preparation.

As shown in Figure 10, the dose response curves for control and diabetic PM receptor kinase activities were similar in shape. However, for this particular preparation, the diabetic receptor kinase activity was greater than the control receptor kinase activity at all concentrations of insulin tested (%D/C= +36 to +64% for [insulin] $\geq$ 1.0 nM).

^{32}P-Orthophosphate Formation in Insulin Receptor Preparations. In view of studies demonstrating changes in the membrane concentration of ion-pump ATPases in diabetes (88,89), the extent of ^{32}P-orthophosphate generation was measured concomittant with measurements of poly Glu/Tyr(4:1) phosphorylation, as described above. The results of these experiments with microsomal and PM preparations are shown in Figures 11 and 12, respectively. In the absence of vanadate and pNPP, the amount of ^{32}P-labelled orthophosphate formed (pmol/fmol insulin binding/20 min)

Figure 9. **EFFECT OF [INSULIN] ON INSULIN RECEPTOR TYROSINE KINASE ACTIVITY IN MICROSOMES**

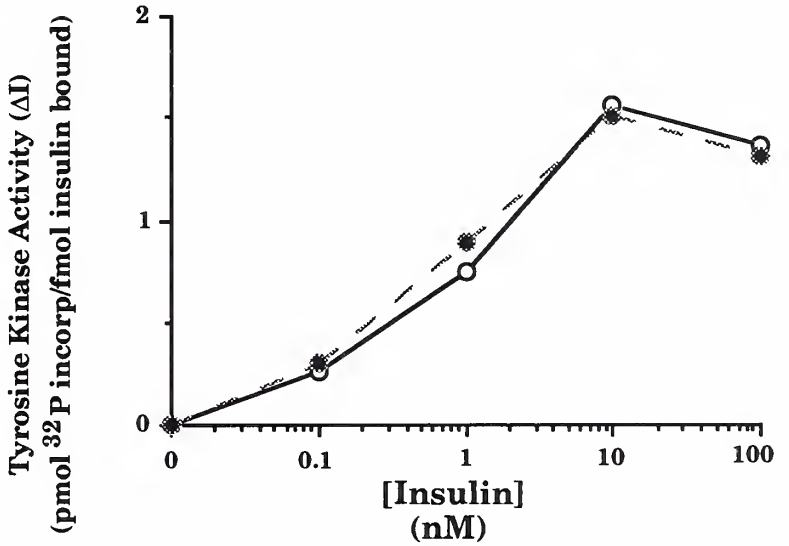
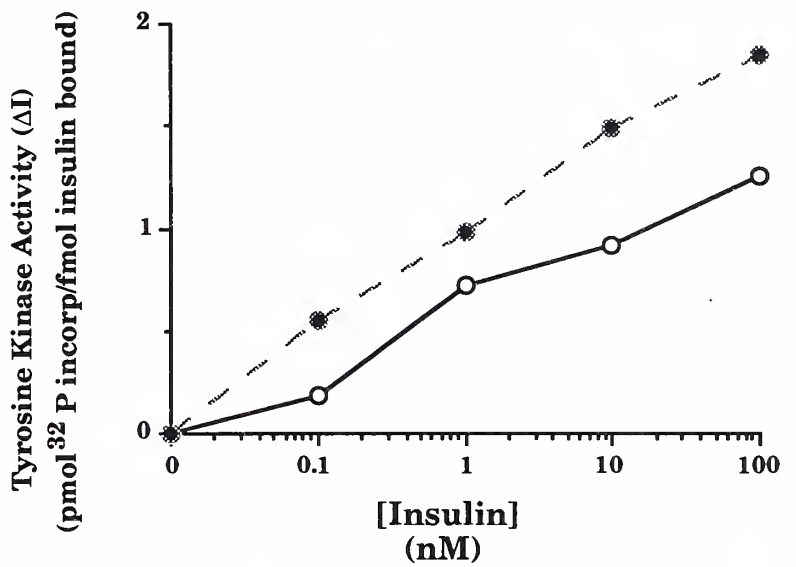


Figure 10. **EFFECT OF [INSULIN] ON INSULIN RECEPTOR TYROSINE KINASE ACTIVITY IN PM**



Figures 9,10. Effect of insulin concentration on insulin receptor tyrosine kinase activity in microsomal (Figure 9) and PM (Figure 10) preparations. As described in Methods, aliquots of lectin-purified hepatic microsomal and PM insulin receptors from normal (open circles) and diabetic (solid circles) rats were incubated in the presence of [γ ^{32} P]ATP (100 μM), pNPP (10 mM), vanadate (0.1mM), and varying concentrations of insulin (0 to 100 nM). Basal tyrosine kinase activity in microsomes was 0.76 (control) and 0.81 (diabetic) (pmol^{32} P incorporated/fmol insulin bound), while it was 0.99 in control and 1.05 in diabetic from PM preparations. Data represent the mean values from one (microsomal) or four replicate (PM) experiments performed in quadruplicate.

was 74 (9.0% hydrolysis) and 45 (5.1% hydrolysis) in insulin receptor preparations from microsomes of normal and diabetic rats, respectively (Figure 11) and was 313 (23% hydrolysis) and 247 (18% hydrolysis) in insulin receptor preparations from PM of normal and diabetic rats, respectively (Figure 12). In the presence of the vanadate and pNPP, an 88% decrease in ^{32}P-orthophosphate levels was found in control microsomal insulin receptor preparations (Figure 11: decreased to 8.9 [1.1% hydrolysis]), while an 87% decrease was seen in diabetic microsomal insulin receptor preparations (Figure 11: decreased to 5.8% [0.7% hydrolysis]). In contrast, the addition of vanadate to insulin receptor preparations from PM resulted in decreases of only 41% in preparations from normal (Figure 12: decreased to 185 [14% hydrolysis]) and 61% in preparations from diabetic (Figure 12: decreased to 96 [7.1% hydrolysis]) rats.

The generation of ^{32}P-orthophosphate was less in the diabetic preparation than in the control preparation for each set of experimental conditions. In the microsomal insulin receptor preparations, the level of ^{32}P-orthophosphate formation was 39% or 35% less in the diabetic group than in the control group when incubated in the absence or presence of vanadate and pNPP, respectively. Similarly, in the PM insulin receptor preparations, the diabetic group exhibited 21% less ^{32}P-orthophosphate formation than the control group in the absence of vanadate and 48% less than the control group in the presence of vanadate.

These results suggested that ATPase and/or phosphatase activity was present in all preparations of insulin receptors tested. This activity was 4 to 21 times greater at all experimental conditions in preparations of PM origin than in preparations from microsomes. Furthermore, vanadate inhibited this ATPase and/or phosphatase activity but to a greater extent in

Figure 11. **^{32}P-ORTHOPHOSPHATE FORMATION IN INSULIN RECEPTOR PREPARATIONS FROM MICROSOMES**

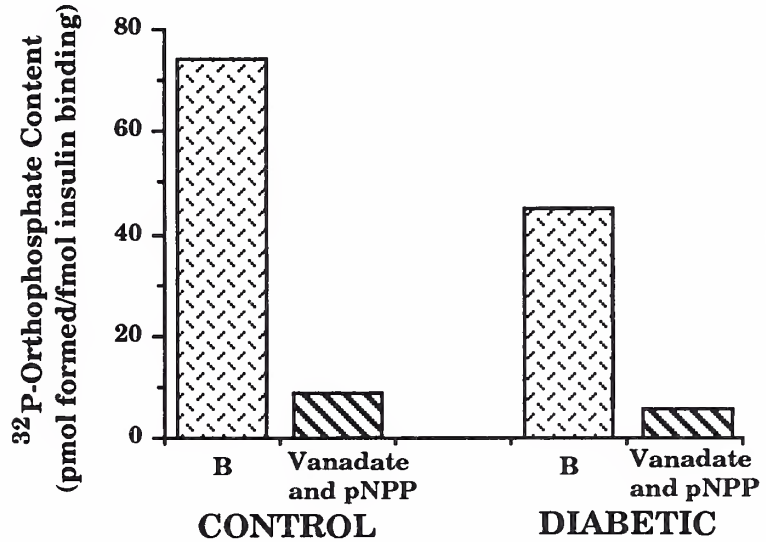
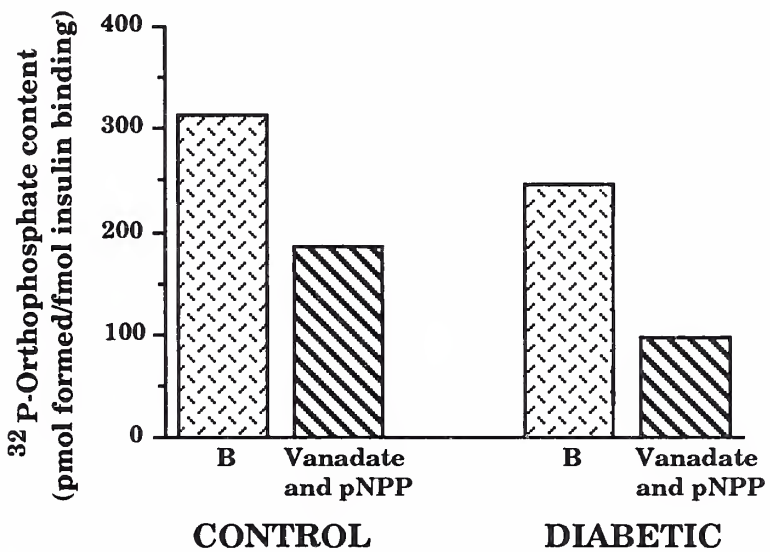


Figure 12. **^{32}P-ORTHOPHOSPHATE FORMATION IN INSULIN RECEPTOR PREPARATIONS FROM PM**



Figures 11,12. **^{32}P-orthophosphate formation in insulin receptor preparations. Aliquots of lectin-purified hepatic microsomal (Figure 11) and PM (Figure 12) insulin receptors from control and diabetic rats were incubated in the presence of 100 μM [γ^{32} P]ATP, 100 nM insulin, poly Glu/Tyr(4:1), and kinase buffer without (B) or with pNPP (10 mM) and vanadate (1 mM). Samples from aliquots were subsequently assayed for ^{32} P-orthophosphate content as described in Methods (20 minutes of hydrolysis). Data represent the mean values from two experiments performed in quadruplicate.**

insulin receptor preparations from microsomes than in those from PM. These ATPases and/or phosphatases were also found to be less active in comparable preparations from diabetic rats than those from normal rats.

Determination of the Effect of ATP Concentration on Insulin Receptor Tyrosine Kinase Activity. In order to assess the activity of the insulin receptor tyrosine kinase without the confounding factor of copurifying ATPase/phosphatase activity, tyrosine kinase activity was evaluated when at multiple ATP concentrations. These experiments were conducted by varying the ATP concentrations (25 to 400 μM) and using double reciprocal plots (Lineweaver-Burke) for analysis of tyrosine kinase activity in order to determine the V_{max} and K_m for the insulin receptor kinase from normal and diabetic preparations in the presence of 1 mM vanadate and 10 mM pNPP as described above. As shown in Figures 13 and 14, the V_{max} was reduced by 42% in the diabetic preparation from microsomes (Figure 13: 6.80 and 3.97 pmol ^{32}P incorporated/fmol insulin binding for control and diabetic preparations, respectively) and by 43% in the diabetic group from PM (Figure 14: 6.37 and 3.64 pmol ^{32}P incorporated/fmol insulin binding for control and diabetic preparations, respectively). The relationship of greater kinase activity in the control group remains intact at all ATP concentrations tested in microsomal preparations, but in PM preparations this relationship inverts at ATP concentrations less than about 150 μM.

These ATPase/phosphatase activities were also consistent with an observed diabetic K_m (Figure 14: 45 μM ATP) which is 76% less than the control K_m (Figure 14: 190 μM ATP) in PM preparations. Higher ATPase/phosphatase activity in the control group would increase the K_m. In microsomes where the ATPase/phosphatase activities are not so disparate,

Figure 13. **EFFECT OF [ATP] ON INSULIN RECEPTOR TYROSINE KINASE ACTIVITY FROM MICROSOMES**

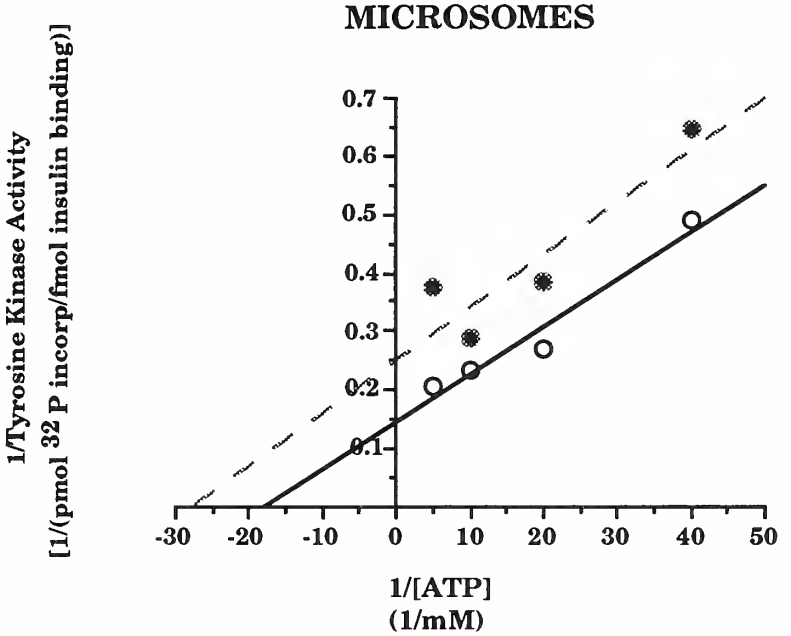
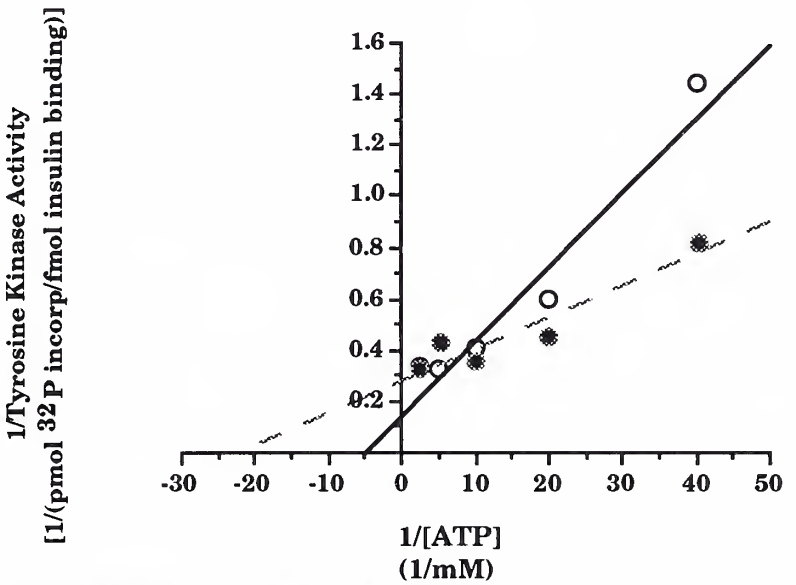


Figure 14. **EFFECT OF [ATP] ON INSULIN RECEPTOR TYROSINE KINASE ACTIVITY FROM PM**



Figures 13,14. The effect of ATP concentration on insulin receptor tyrosine kinase activity in PM and microsome preparations. Aliquots of lectin-purified hepatic PM and microsomal insulin receptors from control (open circles) and diabetic (solid circles) rats were incubated in the presence of 100 nM insulin, 25 to 400 μM [γ^{32}P]ATP, and poly Glu/Tyr(4:1) as described in Methods. The data are expressed in double reciprocal plots and represent the mean values from one experiment performed in quadruplicate.

the diabetic K_m was still 35% less than the control (Figure 14: 36 and 55 μM ATP in diabetic and control preparations, respectively). These results are consistent with previous studies in which the K_m of ATP for the insulin receptor tyrosine kinase was found to range from 30 to 150 μM ATP (reviewed in 8).

Discussion

A major interest of this laboratory has been to explore the mechanisms underlying hepatic insulin resistance. With regard to the insulin resistance of nonketotic diabetes mellitus, this laboratory has previously reported (44) that PM insulin receptor autophosphorylation in response to insulin was normal in streptozotocin-induced diabetic rat livers. It was concluded that the hepatic insulin resistance of nonketotic diabetes mellitus resides distal to insulin receptor binding and autophosphorylation. The present study extended the earlier work by this laboratory (44) by examining the tyrosine kinase activity (toward poly Glu/Tyr[4:1]) of hepatic insulin receptors prepared from the PM of normal and streptozotocin-diabetic rats. For comparison, insulin receptors were prepared from hepatic microsomes and were analyzed.

Experiments testing insulin binding were conducted in order to equalize binding between control and diabetic insulin receptor preparations and to confirm previous observations of insulin binding in microsomes and PM. The finding of increased binding in microsomal insulin receptors isolated from diabetic rats (Figure 1) was consistent with the results of earlier studies (30,39,41,43 48,49). In contrast, one study using a different rat model of diabetes did find reduced insulin binding in diabetic microsomal receptors (35). Experiments with PM preparations (Figure 2) revealed no difference in insulin binding activity between control and diabetic receptors and confirmed the observations made by this laboratory (44) and other investigators (38,45,51,68). It is unclear why microsomal insulin binding increases in diabetes while PM insulin binding remains

unchanged. Perhaps insulin receptor degradation decreases in diabetes leading to receptor accumulation in the microsomal compartment.

Experiments were performed to evaluate the effect of insulin concentration on insulin receptor tyrosine kinase activity (Figures 9,10). Because receptor kinase activity was enhanced at all insulin concentrations tested (0.1 to 100 nM), results from the present study that were obtained using 100 nM insulin were the result of a true insulin effect rather than the early stage of a growth factor effect which occurs at insulin concentrations of greater than 1000 nM (6).

Initial experiments measuring tyrosine kinase activity revealed a high degree of variability. The trichloroacetic acid wash procedure was evaluated in order to eliminate the washing of filter papers as a possible source of this variability. TCA washing was found to be quite effective in bringing radioactivity on filter papers to a steady state within three washes while minimizing the degree of [γ^{32}P]ATP "stickiness" (Figure 3). Thus, it is unlikely that inefficient TCA washing of filter papers accounted for the variability seen in the tyrosine kinase assay.

In addition to the variability observed in the assay, insulin-stimulated tyrosine kinase activity in PM receptors was minimal in initial experiments. The type of buffer was investigated as a possible source of this blunting of kinase activity. Tris buffer had no direct effect on insulin binding but did seem to inhibit kinase activity in microsomal insulin receptors (Table 1). So, in addition to Tris affecting insulin receptor recycling in intact cells (69,70), the current results show a direct inhibitory effect on insulin receptor tyrosine kinase activity. The mechanism underlying this inhibitory effect of Tris is unclear at the present time.

Just as initial tyrosine kinase results were variable in the present study, the literature is filled with inconsistencies with regard to hepatic insulin receptor tyrosine kinase measurements. Previous investigations examining hepatic insulin receptor tyrosine kinase in diabetes have pointed towards alterations, but the results have been contradictory (29,30,33,35,38, 39,41,43-45,48,49). If the methodologies used in these studies are compared, one observes that in all (30,39,45,48,49) but one study (44) receptor tyrosine kinase activity was reduced in the diabetic groups when insulin receptor preparations were incubated with [γ^{32}P]ATP at 4°C. In contrast, when insulin receptor preparations were incubated at ambient temperatures (20-24°C) and in the absence of vanadate, a majority of studies (29,35,49) demonstrated no change or even enhanced receptor tyrosine kinase activity in diabetic preparations. In a study examining tyrosine kinase activity (toward angiotensin-I) in insulin receptor preparations made from rat skeletal muscle, Burant *et al.* (42) described reduced activity in diabetic preparations at incubation temperatures of 25° and 4°C with insulin-agarose bound (purified) and non-immobilized (partially purified) insulin receptors, respectively. This finding would suggest that the observed inconsistencies between phosphorylation studies performed at 4°C (30,39, 45,48,49) and 20-24°C (29,35,49) could have been the result of "contaminating" activities (ATPases/ phosphatases?) which are active at ambient temperatures. These activities are apparently absent in more highly purified preparations of the insulin receptor (43,56,74,79).

CTP has been employed as a competitive inhibitor of contaminating ATPases (31,36,38,50,71,74). In the majority of these studies (36,38,50,71,74), the addition of CTP led to enhanced insulin receptor tyrosine kinase activity. In light of these results CTP was added to the tyrosine kinase

reaction mixture in an attempt to eliminate variability and enhance the minimal insulin response by inhibiting ATPases that might be copurifying with the insulin receptor. Unexpectedly, CTP actually reduced insulin-stimulated tyrosine kinase activity in both control and diabetic preparations (Table 2). The mechanism of this effect was not further investigated, but since CTP has not been shown previously to inhibit the insulin receptor tyrosine kinase, a direct effect is unlikely.

A number of studies have used vanadate in an attempt to inhibit contaminating phosphotyrosine phosphatases and ATPases (31,33,36,38, 47,49,50,71,74,79). A majority of studies (31,38,49,50,71,74) found vanadate to be effective in this role, so the effect of vanadate was evaluated in the present study. Vanadate was shown to enhance insulin-stimulated tyrosine kinase activity in both microsomal (Figure 5) and PM (Figure 6) preparations suggesting the presence of ATPases/phosphatases sensitive to vanadate. In microsomes, vanadate actually enhanced tyrosine kinase activity to a greater extent in control preparations than in diabetic preparations suggesting more ATPase/phosphatase activity copurified with the control group.

Experiments that examined the time course of insulin-stimulated tyrosine kinase activity with and without vanadate yielded similar results. In microsomes without vanadate (Figure 7), kinase activity plateaued earlier in the control than in the diabetic group, but when vanadate was added kinase activity was nearly linear for up to 20 minutes in both groups. These findings suggested again the presence of vanadate-sensitive ATPases/phosphatases in microsomal preparations with more activity in the control group. In PM preparations, in the presence of vanadate insulin-stimulated kinase activity was nearly linear over the first 10

minutes of incubation but plateaued in control preparations by 20 minutes, again implicating more ATPase/phosphatase activity or a greater vanadate-insensitive component in the control group.

With the previous experiments implicating the presence of contaminating ATPases/phosphatases, the actual combined activities of these enzymes were assessed by measuring their by-product, ^{32}P-orthophosphate (from the hydrolysis of [γ^{32}P]ATP by ATPases or from dephosphorylation of ^{32}P-labelled insulin receptor by phosphatases). These data confirmed the existence of contaminating ATPases/phosphatases in both microsomal (Figure11) and PM (Figure 12) preparations. ATPase/ phosphatase activities were found to be greater in control than in diabetic preparations from both membrane compartments. This activity was also significantly inhibited by vanadate in all preparations, however to a lesser degree in the PM preparations, suggesting a vanadate-insensitive component.

In a continuation of the present study, this laboratory evaluated dephosphorylation of ^{32}P-labelled β-subunits of the insulin receptor and found this activity to be higher in insulin receptor preparations from control PM compared to diabetic PM (90). This result is in contrast to previous studies in which microsomal phosphatase activity was found to be unchanged (54) or increased (55) by diabetes. A residual vanadate-insensitive phosphatase was also detected, and preliminary characterization showed it to be a phosphotyrosine phosphatase (90). Previous studies that have isolated phosphatases capable of dephosphorylating the phosphorylated forms of the insulin receptor (52-57,59) or poly Glu/Tyr(4:1) (64,67) have shown this activity to be inhibited by vanadate at doses (1 mM) equal to or less than that used in the present and following

(90) studies. However, vanadate-insensitive phosphotyrosine phosphatases have been isolated (77,78). The function of the phosphatases and ATPases present in the current study and their alterations in diabetes merit further investigation.

The extent of ATPase/phosphatase contamination found in insulin receptor preparations from microsomes appears to be determined by the amount of protein used in incubations to measure insulin receptor tyrosine kinase activity. For example, in microsomes (Figure 11) the ATPase/ phosphatase activity based on ^{32}P-orthophosphate formation per unit insulin binding is 64% higher in the control than in the diabetic preparations but only 30% higher based on activity per unit protein. This finding may explain the observation of Kadowaki *et al.* (43) who reported ATP hydrolysis to be higher (12.5% compared to 4.5% at 4^{o}C) in microsomal insulin receptor preparations from control rats compared to those from diabetic rats. The fact that ATPase/phosphatase activity seems to vary with protein content stresses the importance of monitoring this copurifying activity when comparing preparations of equal binding but different protein content (e.g. microsomal preparations).

Insulin receptor tyrosine kinase activity was examined when ATP concentration was not limiting and in the presence of vanadate in order to eliminate the effect of copurifying ATPases/phosphatases. In microsomal preparations (Figure 13), double reciprocal plot analysis of the data revealed a V_{max} for the diabetic group which was 42% less than that of the control group in agreement with a continuation of the present study by this laboratory (56% reduction in the diabetic V_{max}) (90). This finding of reduced tyrosine kinase activity in the diabetic group confirmed the results of a majority of previous studies evaluating tyrosine kinase activity at sub-

maximal (11,30,32,34, 39,41,43,45,48,49) and maximal (11,32,34) ATP concentrations.

Double reciprocal plot analysis of data from PM preparations (Figure 14) found a V_{max} for the diabetic group which was 43% less than that of the control group. This finding was in agreement with the continuation of the present study by this laboratory (38% reduction in diabetic V_{max}) (90) but was in contrast to the previous study by this laboratory (44) which found that diabetes did not affect insulin-stimulated insulin receptor autophosphorylation. A possible explanation is that autophosphorylation and phosphorylation of other substrates are uncoupled in diabetes. On the other hand, this discrepancy might be explained by the intersecting lines of the double reciprocal plot (Figure 14). In the presence of the lowest concentration of ATP tested (25 μM), the insulin-sensitive tyrosine kinase activity was found to be 77% higher in insulin receptor preparations from diabetic rats, whereas at V_{max}, tyrosine kinase activity was 43% lower in the diabetic group. The greater vanadate-insensitive ATPase activity observed in the control preparation (discussed above, Figure 12) probably contributed greatly to these observations. At low ATP concentrations, the disparate ATPase/phosphatase activities would reduce the measurable kinase activity significantly more in the control than in the diabetic preparations so that the diabetic kinase activity would seem greater than or equal to the control activity. At high ATP concentrations, the effect of ATPase/phosphatase activity would be relatively insignificant, and the true kinase activities of the insulin receptors would be observed.

In light of these findings, the ATPases/phosphatases present in PM preparations but not completely inhibited by vanadate probably confounded the earlier experiments (Figure 6) conducted at submaximal ATP (100 μM)

in which it was concluded that PM tyrosine kinase activity is higher in the diabetic group. Similarly, these ATPases/phosphatases probably also confounded the results of the previous study by this laboratory (44) because it was conducted in the presence of only 25 μM [γ^{32}P]ATP and in the absence of vanadate. In fact, when this experiment was repeated by this laboratory in the presence of 100 μM [γ^{32}P]ATP and vanadate, auto-phosphorylation was reduced by 52% in the diabetic group (90). These observations also emphasize the need to perform phosphorylation measurements at multiple ATP concentrations, especially when using PM preparations.

Lineweaver-Burke analysis also allowed the determination of the K_m of ATP for the insulin receptor tyrosine kinase. In both microsomal (Figure 13) and PM (Figure 14) preparations, the K_m's for ATP were reduced in the diabetic groups in comparison to the control group. Although the reduction in K_m in diabetic preparations might have been due to changes in the structure or conformation of the insulin receptor (no changes have been observed [41]), reduced levels of ATPase/phosphatase activities in the diabetic preparations are more likely to have been responsible for the current observations. Indeed, the higher K_m (ATP) seen in the PM control preparation suggests that a greater degree of vanadate-insensitive ATPases were present.

Lin (76) has described the presence of a calcium/magnesium ecto-ATPase in detergent solubilized preparations of rat hepatic PM. This activity is not identical to membrane ion-pump ATPases based on insensitivity to specific ion-pump ATPase inhibitors, such as vanadate, and the lack of substrate specificity toward ATP. Although the function of this ecto-ATPase is not known, Lin (76) suggested that it may function to

regulate extracellular ATP levels. An ATPase/phosphatase activity was found in this study in both PM and microsomal insulin receptor preparations and found to be only partially suppressible in the former by 1 mM vanadate. Further studies (90) have shown the presence of a nucleotide hydrolyzing activity in PM preparations capable of utilizing GTP, insensitive to vanadate (1 mM), EGTA, and ouabain, and dependent on magnesium or calcium for activation. Indeed, the ATP hydrolyzing activity in the present study may be related to Lin's ecto-ATPase (76) and seems to be altered by diabetes in some way that reduces its concentration and/or activity.

The results of the present study and its continuation by this laboratory (90) seem to suggest that a defect in insulin receptor function at the level of the tyrosine kinase is present in diabetes and cannot be explained by changes in ATPase/phosphatase activity. In fact, more ATPase/phosphatase activity was detected in the control preparations implying that insulin receptor tyrosine kinase activity is reduced by diabetes even more than observed in the present study because of the presence of vanadate-insensitive ATPase/phosphatase activity. The present study is the first to show that this reduction of tyrosine kinase activity by diabetes is also present in PM insulin receptors.

The mechanism(s) underlying this alteration in insulin receptor function by diabetes is unclear. One investigator (36) has suggested that diabetes increased the K_m (ATP) of the insulin receptor kinase, but the results of the present study show the K_m (ATP) to be reduced by diabetes. Other studies have suggested that changes in phosphotyrosine phosphatases are responsible, but the current and following (90) studies found greater phosphatase activity in the control group. Structural or confor-

mational changes in the insulin receptor secondary to alterations in maturation or glycosylation have been suggested (36,40,41) but not been confirmed (40). There is some evidence (11,14,29,36) for a defective auto-activation cascade in the diabetic insulin receptor leading to inadequate signal coupling. Another theory is that diabetes increases serine phosphorylation of the insulin receptor causing it to become inactivated (36,40,41,80). Further research is still needed to define the mechanism(s) underlying the diabetes-induced alterations in the insulin receptor tyrosine kinase before new methods of therapy and prevention can be developed for this common and devastating disease.

References

1. Van Obberghen, E., and S. Gammeltoft. 1986. Insulin receptors: Structure and function. *Experientia* 42:727-734.

2. Goldfine, I.D. 1987. The insulin receptor: Molecular biology and transmembrane signaling. *Endocrine Reviews* 8:235-255.

3. Blackshear, P.J., A.C. Nairn, and J.F. Kuo. 1988. Protein kinases 1988: A current perspective. *FASEB J.* 2:2957-2969.

4. Ebina, Y., E. Araki, M. Taira, F. Shimada, M. Mori, C.S. Craik, K. Siddle, S.B. Pierce, R.A. Roth and W.J. Rutter. 1987. Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin-and antibody-stimulated glucose uptake and recptor kinase activity. *Proc. Nat. Acad. Sci. U.S.A.* 84:704-708.

5. Chou, C.K., T.J. Dull, D.S. Russell, R. Gherzi, D. Lebwohl, A. Ullrich, and O.M. Rosen. 1987. Human insulin receptors mutated at the ATP-binging site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J. Biol. Chem.* 262:1842-1847.

6. Sale, G.J. 1988. Recent progress in our understanding of the mechanism of action of insulin. *Int. J. Biochem.* 20:897-908.

7. Rosen, O.M. 1987. After insulin binds. *Science* 237:1452-1458.

8. Gammeltoft, S., and E. Van Obberghen. 1986. Protein kinase activity of the insulin receptor. *Biochem J.* 235:1-11.

9. King, M.J., and G.J. Sale. 1990. Dephosphorylation of insulin-receptor autophosphorylation sites by particulate and soluble phosphotyrosyl-protein phosphatases. *Biochem J.* 266:251-259.

10. Obermaier-Kusser, B., M.F. White, D.E. Pongratz, Z. Su, B. Ermel, C. Muhlbacher, and H.U. Haring. 1989. A defective intramolecular autoactivation cascade may cause the reduced kinase activity of the skeletal muscle insulin receptor from patients with non-insulin-dependent diabetes mellitus. *J. Biol. Chem.* 264:9497-9504.

11. Kahn, C.R., and M.F. White. 1988. The insulin receptor and the molecular mechanism of insulin action. *J. Clin. Invest.* 82:1151-1156.

12. Moller, D.E., H. Benecke, and J.S. Flier. 1991. Biologic activities of naturally occurring human insulin receptor mutations: Evidence that metabolic effects of insulin can be mediated by a kinase-deficient receptor mutant. *J. Biol. Chem.* 266:10992-11001.

13. Haring, H., and B. Obermaier-Kusser. 1989. Insulin receptor kinase defects in insulin-resistant tissues and their role in the pathogenesis of NIDDM. *Diabetes Metabolism Reviews* 5:431-441.

14. Cherqui, G., C. Reynet, M. Caron, B. Melin, D. Wicek, E. Clauser, J. Capeau, and J. Picard. 1990. Insulin receptor tyrosine residues 1162 and 1163 control insulin stimulation of myristoyl-diacylglycerol generation and subsequent activation of glucose transport. *J. Biol. Chem.* 265:21254-21261.

15. Gawler, D., G. Milligan, A.M. Spiegel, C.G. Unson, and M.D. Houslay. 1987. Abolition of the expression of inhibitory guanine nucleotide regulatory protein G_i activity in diabetes. *Nature, Lond.* 327:229-232.

16. McClain, D.A. 1990. Endocytosis of insulin receptors is not required for activation or deactivation of the hormone response. *J. Biol. Chem.* 265:21363-21367.

17. Obermaier, B., B. Ermel, D. Kirsch, J. Mushack, E. Rattenhuber, E. Biemer, F. Machicao, and H.U. Haring. 1987. Catecholamines and tumour promoting phorbolesters inhibit insulin receptor kinase and induce insulin resistance in isolated human adipocytes. *Diabetologia* 30:93-99.

18. Shechter, Y., P. Yaish, M. Chorev, C. Gilon, S. Braun, and A. Levitzki. 1989. Inhibition of insulin-dependent lipogenesis and anti-lipolysis by protein tyrosine kinase inhibitors. *EMBO J.* 8:1671-1676.

19. Caron, M., G. Cherqui, B. Melin, D. Wicek, J. Capeau, and J. Picard. 1989. Insulin action is mimicked by polyclonal antireceptor antibodies that activate the insulin receptor tyrosine kinase. *Horm. Metab. Res.* 21:295-300.

20. Hawley, D.M., B.A. Maddux, R.G. Patel, K-Y. Wong, P.W. Mamula, G.L. Firestone, A. Brunetti, E. Verspohl, and I.D. Goldfine. 1989. Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. *J. Biol. Chem.* 264:2438-2444.

21. Forsayeth, J.R., J.F. Caro, M.K. Sinha, B.A. Maddux, and I.D. Goldfine. 1987. Monoclonal antibodies to the human insulin receptor that activate glucose transport but not insulin receptor kinase activity. *Proc. Natl. Acad. Sci. U.S.A.* 84:3448-3451.

22. White, M.F., J.N. Livingston, J.M. Backer, V. Lauris, T.J. Dull, A. Ullrich, and C.R. Kahn. 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. *Cell* 54:641-649.

23. Gottschalk, W.K. 1991. The pathway mediating insulin's effects on pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase. *J. Biol. Chem.* 266:8814-8819.

24. Grunberger, G., Y. Zick, and P. Gorden. 1984. Defect in phosphorylation of insulin receptors in cells from an insulin-resistant patient with normal insulin binding. *Science* 223:932-934.

25. Taylor, S.I., T. Kadowaki, H. Kadowaki, D. Accili, A. Cama, and C. McKeon. 1990. Mutations in insulin-receptor gene in insulin-resistant patients. *Diabetes Care* 13:257-279.

26. Kusari, J., Y. Takata, E. Hatada, G Freidenberg, O. Kolterman, and J.M. Olefsky. 1991. Insulin resistance and diabetes due to different mutations in the tyrosine kinase domain of both insulin receptor gene alleles. *J. Biol. Chem.* 266:5260-5267.

27. Moller, D.E., A, Yokota, M.F. White, A.G. Pazianos, and J.S. Flier. 1990. A naturally occurring mutation of insulin receptor alanine 1134 impairs tyrosine kinase function and is associated with dominantly inherited insulin resistance. *J. Biol. Chem.* 265:14979-14985.

28. Taira, M., M. Taira, N. Hashimoto, F. Shimada, Y. Suzuki, A. Kanatsuka, F. Nakamura, Y. Ebina, M. Tatibana, H. Makino, and S. Yoshida. Human diabetes associated with a deletion of the tyrosine kinase domain of the insulin receptor. *Science* 245:63-68.

29. Hurrell, D.G., O. Pedersen, and C.R. Kahn. 1989. Alterations in the hepatic insulin receptor kinase in genetic and acquired obesity in rats. *Endocrinology* 125:2454-2462.

30. Slieker, L.J., E.F. Roberts, W.N. Shaw, and W.T. Johnson. 1990. Effect of streptozotocin-induced diabetes on insulin-receptor tyrosine kinase activity in obese Zucker rats. *Diabetes* 39:619-625.

31. Simon, J., R.W. Rosebrough, J.P. McMurtry, N.C. Steele, J. Roth, M. Adamo, and D. LeRoith. 1986. Fasting and refeeding alter the insulin receptor tyrosine kinase in chicken liver but fail to affect brain insulin receptors. *J. Biol. Chem.* 261:17081-17088.

32. Freidenberg, G.R., H.H. Klein, R. Cordera, and J.M. Olefsky. 1985. Insulin receptor kinase activity in rat liver: Regulation by fasting and high carbohydrate feeding. *J. Biol. Chem.* 260:12444-12453.

33. Blackshear, P.J., R.A. Nemenoff, and J. Avruch. 1984. Characterization of insulin and epidermal growth factor stimulation of receptor autophosphorylation in detergent extracts of rat liver and transplantable rat hepatomas. *Endocrinology* 114:141-152.

34. Arsenis, G., and J.N. Livingston. 1986. Alterations in the tyrosine kinase activity of the insulin receptor produced by in vitro hyperinsulinemia. *J. Biol. Chem.* 261:147-153.

35. Adamo, M., J. Shemer, M. Aridor, J. Dixon, N. Carswell, S.J. Bhathena, O.E. Michaelis, IV, and D. LeRoith. 1989. Liver insulin receptor tyrosine kinase activity in a rat model of type II diabetes mellitus and obesity. *J. Nutr.* 119:484-489.

36. Freidenberg, G.R., R.R. Henry, H.H. Klein, D.R. Reichart, and J.M. Olefksy. 1986. Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetes. *J. Clin. Invest.* 79:240-250.

37. Sinha, M.K., W.J. Pories, E.G. Flickinger, D. Meelheim, and J.F. Caro. 1987. Insulin-receptor kinase activity of adipose tissue from morbidly obesity humans with and without NIDDM. *Diabetes* 36:620-625.

38. Kergoat, M., J. Simon, and B. Portha. 1988. Insulin binding and insulin receptor tyrosine kinase activity are not altered in the liver of rats with non-insulin-dependent diabetes. *Biochem. Biophys. Res. Commun.* 152:1015-1022.

39. Gherzi, R., G. Andraghetti, E. Ferrannini, and R. Cordera. 1986. Insulin receptor autophosphorylation and kinase activity in streptozotocin diabetic rats: Effects of a short fast. *Biochem. Biophys. Res. Commun.* 140:850-856.

40. Brillon, D.J., G.R. Freidenberg, R.R. Henry, and J.M. Olefksy. 1989. Mechanism of defective insulin-receptor kinase activity in NIDDM: Evidence for two receptor populations. *Diabetes* 38:397-403.

41. Okamoto, M., M.F. White, R. Maron, and C.R. Kahn. 1986. Autophosphorylation and kinase activity of insulin receptor in diabetic rats. *Am. J. Physiol.* 251:E542-E550.

42. Burant, C.F., M.K. Truetelaar, and M.G. Buse. 1986. Diabetes-induced and functional changes and structural changes in insulin receptors from skeletal muscle. *J. Clin. Invest.* 77:260-270.

43. Kadowaki, T., M. Kasuga, Y. Akanuma, O. Ezaki, and F. Takaku. 1984. Decreased autophosphorylation of the insulin receptor kinase in streptozotocin-diabetic rats. *J. Biol. Chem.* 259:14208-14216.

44. Amatruda, J.M., and A.M. Roncone. 1985. Normal hepatic insulin receptor autophosphorylation in nonketotic diabetes mellitus. *Biochem. Biophys. Res. Commun.* 129:163-170.

45. Caro, J.F., O. Ittoop, W.J. Porfies, D. Meelheim, E.G. Flickinger, F. Thomas, M. Jenquin, J.F. Silverman, P.G. Khazaire, and M.D. Sinha. 1986. Studies on the mechanism of insulin resistance in the liver from humans with non-insulin-dependent diabetes. *J. Clin. Invest.* 78:249-258.

46. Camps, M., A. Guma, X. Testar, M. Palacin, and A. Zorzano. 1990. Insulin resistance of skeletal muscle during pregnancy is not a consequence of intrinsic modifications of insulin receptor binding or kinase activities. *Endocrinology* 127:2561-2570.

47. Comi, R.J., G. Grunberger, and P. Gorden. 1987. Relationship of insulin binding and insulin-stimulated tyrosine kinase activity is altered in type II diabetes. *J. Clin. Invest.* 79:453-462.

48. Oshima, E., K. Watanabe, and I. Makino. 1990. Decreased autophosphorylation and kinase activity of insulin receptor in diabetic Chinese hamsters. *Diabetes Research Clinical Practice* 10:35-43.

49. Block, N.E., K. Komori, K.A. Robinson, S.L. Dutton, C.F. Lam, and M.G. Buse. 1991. Diabetes-associated impairment of hepatic insulin receptor tyrosine kinase: A study of mechanisms. *Endocrinology* 128:312-322.

50. Nyomba, B.L., V.M. Ossowski, C. Bogardus, and D.M. Mott. 1990. Insulin-sensitive tyrosine kinase: Relationship with in vivo insulin action in humans. *Am. J. Physiol.* 258:E964-E974.

51. Grunberger, G., K. Nagy, D. Rempinski, and J. Levy. 1990. Abnormal insulin receptor tyrosine kinase activity in kidney basolateral membranes from non-insulin-dependent diabetic rats. *J. Lab. Clin. Med.* 115:704-711.

52. King, M.J., and G.J. Sale. 1988. Assay of phosphostyrosyl protein phosphatase using synthetic peptide 1142-1153 of the insulin receptor. *FEBS Letters* 237:137-140.

53. King, M.J., and G.J. Sale. 1988. Insulin-receptor phosphotyrosyl-protein phosphatases. *Biochem. J.* 256:893-902.

54. Gruppuso, P.A., J.M. Boylan, B.I. Posner, R. Faure, and D.L. Brautigan. 1990. Hepatic protein phosphotyrosine phosphatase: Dephosphorylation of insulin and epidermal growth factor receptors in normal and alloxan diabetic rats. *J. Clin. Invest.* 85:1754-1760.

55. Meyerovitch, J., J.M. Backer, and C.R. Kahn. 1989. Hepatic phosphotyrosine phosphatase activity and its alteration in diabetic rats. *J. Clin. Invest.* 84:976-983.

56. Kowalski, A., H. Gazzano, A. Fehlmann, and E. Van Obberghen. 1983. Dephosphorylation of the hepatic insulin receptor: Absence of intrinsic phosphatase activity in purified receptors. *Biochem. Biophys. Res. Commun.* 117:885-893.

57. Roome, J., T. O'Hare, P.F. Pilch, and D.L. Brautigan. 1988. Protein phosphotyrosine phosphatase purified from the particulate fraction of human placenta dephosphorylates insulin and growth-factor receptors. *Biochem J.* 256:493-500.

58. Meyerovitch, J., J.M. Backer, and C.R. Kahn. 1989. Insulin responsive phosphotyrosine phosphatases: A possible regulatory mechanism of insulin receptor action. *Diabetes* 38:41A.

59. Strout, H.V., P.P. Vicario, R. Saperstein, and E.E. Slater. 1988. A protein phosphotyrosine phosphatase distinct from alkaline phosphatase with activity against the insulin receptor. *Biochem. Biophys. Res. Commun.* 151:633-640.

60. Chan, C.P., S.J. McNall, E.G. Krebs, and E.H. Fischer. 1988. Stimulation of protein phosphatase activity by insulin and growth factors in 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A.* 85:6257-6261.

61. Olivier, A.R., L.M. Ballou, and G. Thomas. 1988. Differential regulation of S6 phosphorylation by insulin and epidermal growth factor in Swiss mouse 3T3 cells: Insulin activation of type 1 phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* 85:4720-4724.

62. Leis, J.F., A.F. Knowles, and N.O. Kaplan. 1985. Demonstration of separate phosphotyrosyl- and phosphoseryl-histone phosphatase activities in the plasma membranes of a human astrocytoma. *Arch. Biochem. Biophys.* 239:320-326.

63. Swarup, G., K.V. Speeg, Jr., S. Cohen, and D.L. Garbers. 1982. Phosphotyrosyl-protein phosphatase of TCRC-2 cells. *J. Biol. Chem.* 257:7298-7301.

64. Okada, M., K. Owada, and H. Nakagawa. 1986. Phosphotyrosine protein phosphatase in rat brain: A major phosphotyrosine protein phosphatase is a 23 kDa protein distinct from acid phosphatase. *Biochem J.* 239:155-162.

65. Li, H.C., J. Chernoff, L.B. Chen, and A. Kirschonbaum. 1984. A phosphotyrosyl-protein phosphatase activity associated with acid phosphatase from human prostate gland. *Eur. J. Biochem.* 138:45-51.

66. Swarup, G. and G. Subrahmanyam. 1989. Purification and characterization of a protein-phosphotyrosine phosphatase from rat spleen which dephosphorylates and inactivates a tyrosine-specific protein kinase. *J. Biol. Chem.* 264:7801-7808.

67. Lim Tung, H.Y., and L.J. Reed. 1987. Identification and purification of a cytosolic phosphotyrosyl protein phosphatase from bovine spleen. *Anal. Biochem.* 161:412-419.

68. Salhanik, A.I., H. West, and J.M. Amatruda. 1985. The mechanisms of up-regulation of the hepatic insulin receptor in hypoinsulinemic diabetes mellitus. *J. Biol. Chem.* 260:16232-16236.

69. Begum, N., H.M. Tepperman, and J. Tepperman. 1986. Effect of Trifluoperazine on the action of insulin in rat adipocytes. *Endocrinology* 118:287-292.

70. Marshall, S. and J.M. Olefsky. 1983. Separate intracellular pathways for insulin receptor recycling and insulin degradation in isolated rat adipocytes. *J. Cell Physiol.* 117:195-203.

71. Brillon, D.J., R.R. Henry, H.H. Klein, J.M. Olefsky, and G.R. Freidenberg. 1988. Functional and structural differences in human and rat-derived insulin receptors: Characterization of the beta-subunit kinase activity. *Endocrinology* 123:1837-1846.

72. Zick, Y., G. Grunberger, R.W. Rees-Jones, and R.J. Comi. 1985. Use of tyrosine-containing polymers to characterize the substrate specificity of insulin and other hormone-stimulated tyrosine kinases. *Eur. J. Biochem.* 148:177-182.

73. Rees-Jones, R.W., J.A. Hedo, Y. Zick, and J. Roth. 1983. Insulin-stimulated phosphorylation of the insulin receptor precursor. *Biochem. Biophys. Res. Commun.* 116:417-422.

74. Simon, J., and D. LeRoith. 1986. Insulin receptors of chicken liver and brain: Characterization of alpha and beta subunit properties. *Europ. J. Biochem.* 158:125-132.

75. Iwasa, T., Y. Iwasa, and R. Krishnaraj. 1983. Comparison of high affinity Ca^{2+}-ATPase in rat liver plasma membranes. *Arch Int. Pharmacodyn.* 264:40-58.

76. Lin, S-H. 1985. The rat liver plasma membrane high affinity (Ca^{2+}-Mg^{2+})-ATPase is not a calcium pump: Comparison with ATP-dependent calcium transporter. *J. Biol. Chem.* 260: 10976-10980.

77. Gresser, M.J., A.S. Tracey, and P.J. Stankiewicz. 1987. The interaction of vanadate with tyrosine kinases and phosphatases. *Adv. Prot. Phosphatases* 4:35-57.

78. Lau, K.H., J.R. Farley, and D.J. Baylink. 1989. Phosphatyrosyl protein phosphatases. *Biochem . J.* 257:23-36.

79. Ridge, K.D., K. Hofmann, and F.M. Finn. 1988. ATP sensitizes the insulin receptor to insulin. *Proc. Natl. Acad. Sci. U.S.A.* 85:9489-9493.

80. Ballotti, R., A. Kowalski, M.F. White, Y. Le Marchand-Brustel, and E. Van Obberghen. 1987. Insulin stimulated tyrosine phosphorylation of its receptor beta-subunit in intact rat hepatocytes. *Biochem J.* 241:99-104.

81. Reinmann, E.M., and R.A. Umfleet. 1978. Selective precipitation of $^{32}P_i$ onto filter papers: Application to ATPase and cyclic AMP phosphodiesterase determination. *Biochem. Biophys. Acta* 523:516-521.

82. Zick, Y., M. Kasuga, C.R. Kahn, and J. Roth. 1983. Characterization of insulin-mediated phosphorylation of the insulin receptor in a cell-free system. *J. Biol. Chem.* 258:75-80.

83. Salhanick, A.I., P. Konowitz, and J.M. Amatruda. 1983. Potentiation of insulin action by a sulfonylurea in primary cultures of hepatocytes from normal and diabetic rats. *Diabetes* 32:206-212.

84. Lesko, L., M. Donlon, G.V. Marinetti, and J.D. Hare. 1973. Rapid method for the isolation of rat liver plasma membranes using an aqueous two-phase polymer system. *Biochim. Biophys. Acta.* 311:173-179.

85. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

86. Zick, Y., J. Whittaker, and J. Roth. 1983. Insinin stimulated phosphorylation of its own receptor. *J. Biol. Chem.* 258:3431-3434.

87. Burant, C.F., M.K. Truetelaar, and M.G. Buse. 1988. Tissue specific differences in the insulin receptor kinase activated in vitro and in vivo. *Endocrinology* 122:427-437.

88. Levy, J., J.R. Gavin, III, M.R. Hammerman, and L.V. Avioli. 1986. $Ca^{2+}Mg^{2+}$-ATPase activity in kidney basolateral membrane in non-insulin-dependent diabetic rats: Effect of insulin. *Diabetes* 35:899-905.

89. Nagy, K., G. Grunberger, and J. Levy. 1990. Insulin antagonistic effects of insulin receptor antibodies on plasma membrane (Ca-Mg)-ATPase activity: A possible etiology of Type B insulin resistance. *Endocrinology* 126:45-52.

90. Salhanick, A.I., D.J. Alaimo, and J.M. Amatruda. Decreased tyrosine kinase activity in partially purified plasma membrane hepatic insulin receptors from diabetic rats and influence of copurifying ATPases and phosphatases. (Submitted).

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